



Research paper

Fluoroindole chalcone analogues targeting the colchicine binding site of tubulin for colorectal oncotherapy

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ABSTRACT

Colorectal cancer (CRC) is a common malignancy of the gastrointestinal tract with high morbidity and mortality. Our previous studies have demonstrated that indole-chalcone-based compounds targeting tubulin displayed potential cytotoxicity to CRC cells. Herein, three new series of derivatives were systematically designed and synthesized to explore their structure-activity relationship (SAR) against CRC based on prior research. Among them, a representative fluorine-containing analog (FC116) exerted superior efficacy on HCT116 ($IC_{50} = 4.52$ nM) and CT26 ($IC_{50} = 18.69$ nM) cell lines, and HCT116-xenograft mice with tumor growth inhibition rate of 65.96% (3 mg/kg). Of note, FC116 could also suppress the growth of organoid models ($IC_{50} = 1.8$ – 2.5 nM) and showed adenoma number inhibition rate of 76.25% at the dose of 3 mg/kg in APC^{min/+} mice. In terms of mechanism, FC116 could induce endoplasmic reticulum (ER) stress to produce excess reactive oxygen species (ROS), leading to mitochondrial damage to promote the apoptosis of CRC cells by targeting microtubules. Our results support that indole-chalcone compounds are promising tubulin inhibitors and highlight the potential of FC116 to combat CRC.

1. Introduction

Colorectal cancer (CRC) is a common malignant tumor, which accounts for over 1.9 million new cases and nearly 100,000 deaths worldwide each year [1]. Once diagnosed as advanced CRC, the prognosis of patients is only two years at most [2]. Currently, although immunotherapy has significantly improved the survival rate of patients, surgery combined with chemotherapy is still the preferred treatment option for CRC. The commonly used first-line drugs are fluorouracil, irinotecan, and oxaliplatin, etc. The side effects of long-term use, such as systemic toxicity and drug resistance, also lead to the poor prognosis [3, 4]. It is urgent to discover a novel drug with high efficiency and little toxic side effects to combat CRC.

Microtubules are involved in the composition of the eukaryotic cytoskeleton and play a vital role in cell division by forming the spindle in the prophase of mitosis [5]. The abnormal cell cycle and proliferation induced by mitosis dysregulation are the common features of tumor cells [6]. One of the effective strategies for the clinical treatment of cancer is to interfere with the mitotic process [7,8]. Therefore, microtubules are regarded as important targets for tumor therapy.

Microtubule-targeting drugs (MTAs) are the most widely used anti-tumor agents in clinic [9–11]. According to the different activities on microtubules, MTAs are classified into two categories: microtubule stabilizers and microtubule destabilizers. Microtubule stabilizers can stabilize microtubules by enhancing the contact of microtubules to disturb the dynamic balance of microtubules, such as paclitaxel (PTX).

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On the contrary, microtubule destabilizers make microtubules unstable by impeding the conformational changes of microtubules, such as colchicine and combretastatin A-4 (CA-4), etc. CA-4 inhibits microtubulin polymerization by targeting colchicine site and has good anti-cancer mitogenic effects, but its susceptibility to *trans*-stilbene isomerization limits its clinical application [12,13]. Clinically, PTX is approved as a first-line drug for a variety of solid tumors [14–17]. However, the acquired drug resistance after long-term use of PTX-based MTAs greatly limits their clinical application [18]. Despite there are many binding sites on microtubules, colchicine binding site inhibitors could overcome drug resistance owing to their simple structures and non-P-glycoprotein substrates [19]. However, colchicine and the compounds targeting colchicine binding site have not been approved for cancer therapy due to the severe cytotoxicity and narrow therapeutic window [20–22].

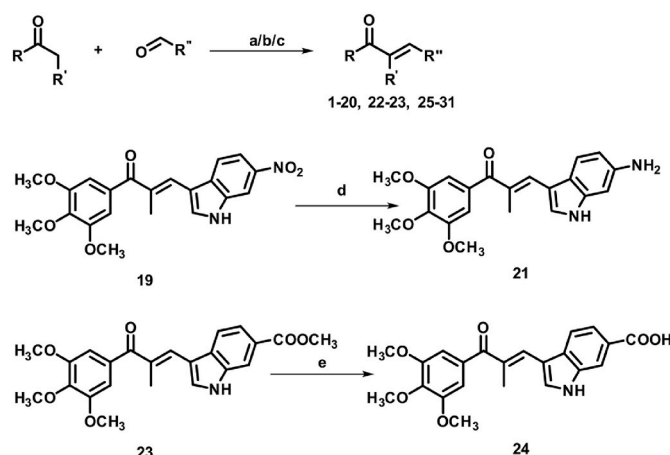
Chalcones are a class of vital flavonoid compounds with privileged structures in medicinal chemistry [25]. Due to their simple structural scaffold and anticancer properties, chalcones can be easily modified with other anticancer pharmacophores to form diverse bioactive derivatives [26]. Moreover, flexible conformation makes chalcones effectively bind a variety of enzymes and receptors [27,28]. We previously found chalcones modified with trimethoxy is easy to bind to tubulin, preventing its polymerization, disrupting mitotic spindle assembly, interfering with cytoskeletal function, inducing the cell cycle arrest and cell apoptosis, which are important mechanisms of anti-proliferative activity [29]. In addition, indole is a nitrogen heterocyclic compound composed of a benzene ring and an N-containing pyrrole ring, which is also a specific pharmacophore [30]. Indole derivatives have become a kind of multi-purpose drug scaffold with good anticancer potential due to their good pharmacokinetic and pharmacodynamic properties [31–33]. Moreover, indole scaffolds can bind to the colchicine binding sites of tubulin and inhibit tubulin polymerization, and numerous tubulin inhibitors based on indole scaffolds have been reported [34–37].

The previously synthesized indole-chalcone compound, (E)-3-(1H-indol-3-yl)-2-methyl-1-(3,4,5-trimethoxyphenyl) prop-2-en-1-one (FC77), showed excellent cytotoxicity against CRC cell lines, but it has high toxicity in mice [29]. Indole-chalcone compound has been reported as an important source of compounds to target the colchicine binding site of microtubules, which can be modified to reduce the cytotoxicity to normal cells and increase the inhibition effect on tumor cells [37,38]. Herein, three series of derivatives of FC77 were designed, and their structure-activity relationship (SAR) against CRC was explored. Furthermore, the inhibition effect and mechanisms of a representative fluorine-containing analog (FC116) for CRC were systematically investigated.

2. Results

2.1. Structure-activity relationship of indole-chalcone compounds against CRC cells

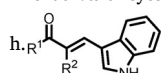
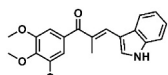
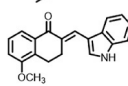
Based on our previous study, the indole moiety and α -methyl substitution were shown to be critical to the cytotoxicity [29]. We developed three series of derivatives taking FC77 as a lead compound to explore the SAR against CRC cell line HCT116. These analogues were prepared and readily converted from an acetophenone and a benzaldehyde by a classic Claisen-Schmidt reaction (Scheme 1). All the derivatives were fully characterized by NMR and MS spectra, and the purities (>95%) were determined by high-performance liquid chromatography (HPLC) analysis for subsequent biological experiments. Firstly, the indole moiety of FC77 was kept and the trimethoxy group on chalcone was changed (Table 1). Compound 1, with the removal of trimethoxy group, displayed a GI_{50} (growth inhibition) much lower than that of FC77 in ~1000-fold. Removing the α -methyl group (compound 2) made the cytotoxicity totally lost even at 10 μ M. Compounds 3–6 were synthesized in order to further evaluate the influence of the trimethoxy



Scheme 1. Synthesis of indole chalcone analogues. Reagents and conditions: (a) ethanol, piperidine, 95 °C, 48 h for 2–9, 14–16, 18–20, 22, 23 and 30 with yields of 39–63%. (b) sodium hydroxide (50 wt%), 95 °C, 48 h for 29 and 31 with yields of 41–54%. (c) toluene, piperidine, Dean-Stark trap, 110 °C, 4 h for 1, 10–13, 17 and 25–28 with yields of 36–57%. (d) ethanol/water (3:1), NH_4Cl , iron powder, 55 °C, 6 h for 21 with a yield of 82%. (e) methanol/water (5:1), sodium hydroxide, rt, 9 h, for 24 with a yield of 93%.

Table 1

The *in vitro* cytotoxicity of analogues (1–9) against HCT116 cells for 48

h.R ¹  R ²			
Compounds	R ¹	R ²	GI ₅₀ (μ M)
FC77			0.00099 $\pm$ 0.00008
1		CH ₃	>15
2		H	>10
3		CH ₃	0.065 $\pm$ 0.010
4		CH ₃	0.074 $\pm$ 0.013
5		CH ₃	0.348 $\pm$ 0.043
6		CH ₃	0.061 $\pm$ 0.008
7		CH ₃	0.240 $\pm$ 0.015
8		CH ₂ CH ₃	0.043 $\pm$ 0.005
9			>10

group on the cytotoxicity. Compound 3 with non-substitution on 4-position showed a GI_{50} of 65 nM. Compound 4 with 3,4-dimethoxy group and compound 5 with 3-methoxy group showed decreased GI_{50} of 80 nM and 348 nM. Making two methoxy group to 1,3-dioxolane (compound 6) kept the activity. These compounds highlighted the importance of the 3-, 5-methoxy groups on the cytotoxicity. Pyridine-containing compound 7 showed a low cytotoxicity against the HCT116 cells. Replacing the α -methyl by ethyl obtained compound 8 exhibiting a GI_{50} of 43 nM. Compound 9 with 3,4-dihydronaphthalenone was inactive.

Next, the trimethoxyphenyl group and α -methyl substitution were

retained and the indole moiety was further evaluated (Table 2). A methyl group was introduced onto the indole (compound 10–13). Compound 12 with 6-methyl group showed the best activity ($GI_{50} = 46$ nM) in this series. Compound 10 with 4-methyl group had the lowest cytotoxicity. Compound 11 with 5-methyl and compound 13 with 7-methyl displayed similar GI_{50} ($GI_{50} = 71$ and 107 nM). Then, compound 14 with 5-fluoro and compound 17 (FC116) with 6-fluoro were synthesized and their cytotoxicities were great with GI_{50} of 29 nM and 79 nM. Compound 15 with 5-chloro was still very potent (HCT116, $GI_{50} = 70$ nM). However, compound 16 with 5-bromo was almost inactive ($GI_{50} = 8.3$ μ M). The strong electron-withdrawing group (NO_2) was introduced to obtain compounds 18–20, possessing high cytotoxicity. The nitro group was reduced to an amino group to obtain compound 21 with keeping the cytotoxicity. Compound 22 exhibited a GI_{50} value of 74 nM. The replaced methoxycarbonyl (compound 23) group made the activity decreased. Compound 24 with a carboxyl group totally lost the cytotoxicity.

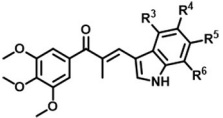
Third, the indole moiety was changed by different heterocycles (Table 3). We changed the benzyl ring of the indole by pyridine to obtain compounds 25–30. Compound 25 with 2-pyridine and compound 28 with 5-pyridine showed better potency in this series. Compound 29 with an imidazo [1,2-a] pyridine group had a low GI_{50} of 71 nM. The nitrogen on the pyridine ring seemed to be very important to the activity. The 3-N-(compound 26), 4-N-pyridine (compound 27) and the pyrazolo [1,5-a] pyridine (compound 30) led dramatically decreased potency. If the nitrogen of the indole was replaced by a sulfur (compound 31), the compound was still active, but less sensitive toward HCT116 cells with a GI_{50} of 200 nM. Therefore, heterocycle substitutions did not show enhanced activities compared to indole.

2.2. FC116 inhibited the proliferation of CRC cells

Three series of derivatives taking FC77 as a lead compound were designed and synthesized (Fig. 1A), and their effects on HCT116 cells were evaluated by CCK-8 assay and SAR was discussed earlier (Tables 1–3). Given above SAR and the role of fluorine substitution on metabolic pathways and pharmacokinetic properties [39], compound 17, also named FC116 (Fig. 1B), which contains fluorine groups, was selected as the lead compound for further evaluation. FC116 exhibited better antiproliferative activity with IC_{50} value of 4.52 nM on HCT116 cells than PTX (IC_{50} value = 23.97 nM) and CA-4 (IC_{50} value = 15.76 nM) respectively (Fig. S1). Meanwhile, the IC_{50} value of FC116 toward

Table 2

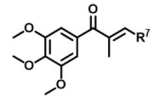
The *in vitro* cytotoxicity of analogues (10–24) against HCT116 cells for 48 h.



Compounds	R ³	R ⁴	R ⁵	R ⁶	GI_{50} (μ M)
10	CH ₃	H	H	H	0.602 $\pm$ 0.025
11	H	CH ₃	H	H	0.071 $\pm$ 0.009
12 [23]	H	H	CH ₃	H	0.046 $\pm$ 0.007
13	H	H	H	CH ₃	0.107 $\pm$ 0.008
14	H	F	H	H	0.029 $\pm$ 0.008
15	H	Cl	H	H	0.070 $\pm$ 0.005
16	H	Br	H	H	>8
17 (FC116)	H	H	F	H	0.079 $\pm$ 0.007
18	H	NO ₂	H	H	0.074 $\pm$ 0.009
19	H	H	NO ₂	H	0.076 $\pm$ 0.004
20	H	H	H	NO ₂	0.091 $\pm$ 0.002
21 [24]	H	H	NH ₂	H	0.069 $\pm$ 0.002
22	H	H	OCH ₃	H	0.074 $\pm$ 0.018
23	H	H	COOCH ₃	H	0.536 $\pm$ 0.148
24	H	H	COOH	H	>10

Table 3

The *in vitro* cytotoxicity of analogues (25–31) against HCT116 cells for 48 h.



Compounds	R ⁷	GI_{50} (μ M)
25		0.049 $\pm$ 0.015
26		0.821 $\pm$ 0.099
27		1.084 $\pm$ 0.146
28		0.059 $\pm$ 0.006
29		0.071 $\pm$ 0.018
30		0.079 $\pm$ 0.005
31		0.200 $\pm$ 0.033

mouse-derived CT26 cells was 18.69 nM. Interestingly, FC116 did not display cytotoxicity on NCM460 cells (normal intestinal epithelial cells) and PC12 cells (neuronal cells) compared to CRC cells at the same dose (Fig. 1C). The effects of FC116 on the viability of HCT116 and CT26 cells were then confirmed visually by colony formation assay and Calcein/PI staining, which showed a significant inhibition effect on cell proliferation (Fig. 1 D-F and Fig. S2A). After FC116 treatment, the cells were stained with PI and Annexin V-FITC, and flow cytometry results showed a significant increase in apoptosis (Fig. 1G–H and Fig. S2 B-C). These results indicate that FC116 had great potential to inhibit the proliferation of CRC cells.

2.3. FC116 suppressed the growth of HCT116 xenograft tumors

We firstly performed *in vivo* pharmacokinetic experiments in mice, As shown in Table S1, FC116 exhibited satisfactory metabolic stability. The further antitumor effect of FC116 was assessed by HCT116 xenograft mouse model. The mice were treated with FC116 by intraperitoneal injection doses of 1.5 and 3 mg/kg per day, and the positive control PTX was administered three times a week at 7 mg/kg for three weeks (Fig. 2A). The treatment with FC116 could significantly inhibit the tumor growth in mice bearing HCT116 tumors, and the inhibitory effect of high-concentration dosing group (3 mg/kg/day) was better than the PTX group. All treated mice showed no significant weight loss or side effects (Fig. 2B–E). Pathological and immunohistochemical analysis of tumor sections showed that FC116 (3 mg/kg/day) resulted in a significant increase in necrotic area, apoptosis and inhibition of cell proliferation compared to the control group (Fig. 2 F and 2G). No major organ-related toxicity by H&E staining in the administered treatment group (Fig. S3). These results showed that FC116 could safely exert powerful antitumor effects *in vivo*.

2.4. FC116 directly bound to microtubules

FC116 has exerted superior inhibition effects on CRC *in vitro* and *in vivo*. To further confirm the binding of FC116 with microtubule, the target prediction was first performed using two different online databases (SEA/Pharmaper), both of which showed that FC116 had the highest score for binding to microtubules (Tables S2 and S3). Cell cycle arrest is a key feature of MTAs [21]. Therefore, the effect of FC116 on

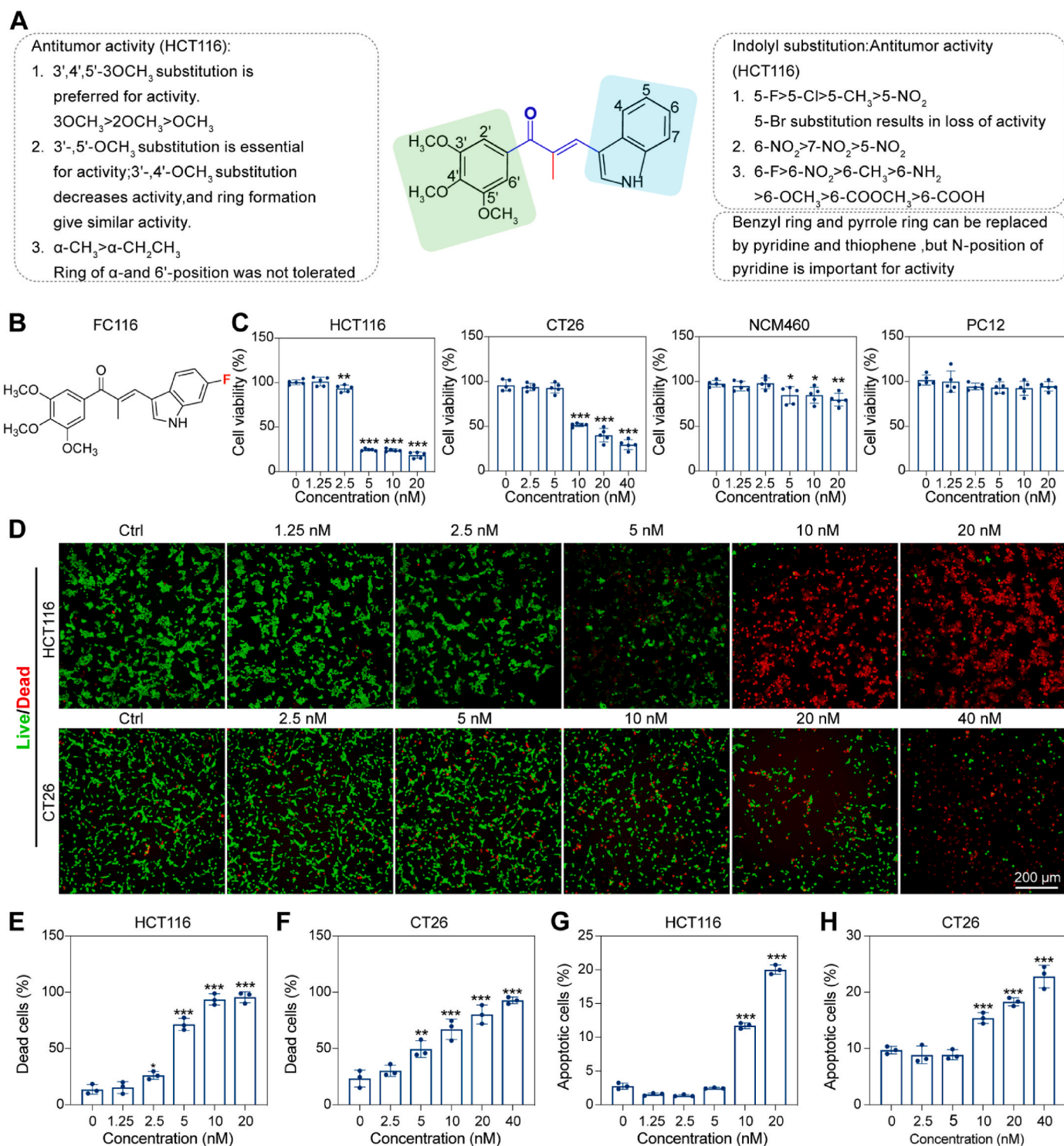


Fig. 1. FC116 inhibited the proliferation and induced apoptosis of CRC cells. (A) Summarized SAR of indole-chalcones. (B) Chemical structure of FC116. (C) HCT116, CT26, NCM460, and PC12 cell proliferation were examined using CCK-8 assay after treatment with FC116 for 24 h. (D–F) Immunofluorescence statistical analysis of FC116-treated HCT116 and CT26 cells after staining with Calcein-AM/PI. (G–H) Cell apoptosis analyzed by flow cytometry staining with PI and Annexin V-FITC, and relative apoptosis rates were assessed. Data were shown as mean $\pm$ S.D. (n = 5), * P < 0.05, ** P < 0.01, *** P < 0.001 versus the control group.

cell cycle in HCT116 and CT26 cells were detected. The results showed that FC116 significantly blocked the cell cycle in the G₂/M phase, which is consistent with the anti-microtubules mechanism of MTAs (Fig. 3A and Fig. S4A).

Then a quantitative proteomics method based on DARTS mass spectrometry (MS) was used for target draping and a variety of different isoforms of microtubules was draped (Table S4). We further validated

those results by western blot assay and found that FC116 significantly increased the stabilization of microtubules during the proteolysis process (Fig. 3 B and C). We then used CETSA to detect whether FC116 could increase the thermal stability of microtubules (Fig. S4B). Unfortunately, the results showed that FC116 did not increase the thermal stability of microtubules, which probably due to the extremely high thermal stability of microtubules.

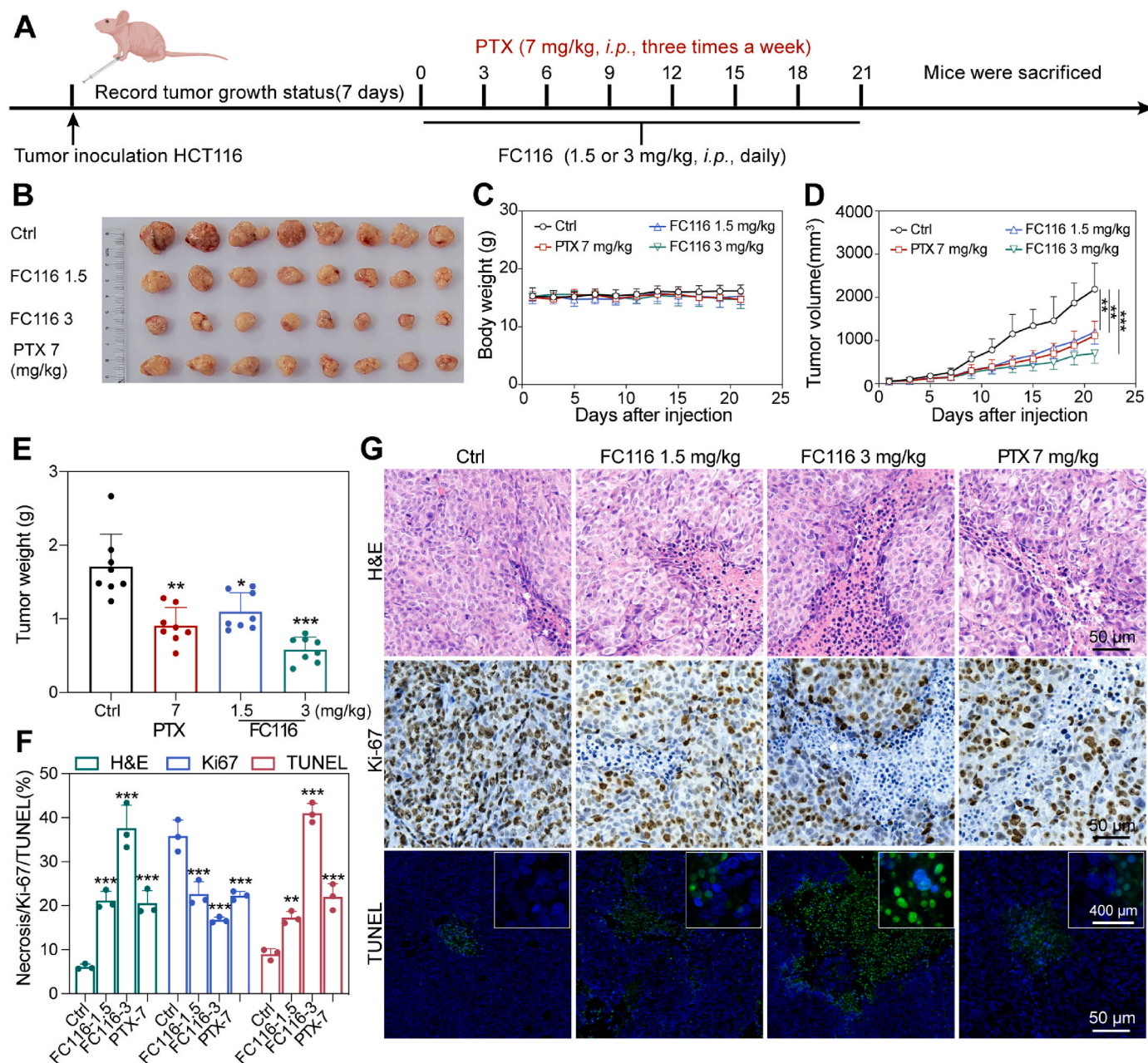


Fig. 2. FC116 inhibited the growth of HCT116 xenograft tumors. (A) Schematic diagram of the planned flow of FC116 administration (1.5/3 mg/kg/day, *i.p.*). (B–E) Mice were monitored for weight and tumor volume every two days until day 21 when they were sacrificed and the excised tumors were photographed and tumor weight was measured. (F–G) Representative stained images of H&E, Ki-67 and TUNEL of tumor tissue sections with quantification of necrotic areas as well as positive tumor cells. Data were shown as mean $\pm$ S.D. (n = 8). * p < 0.05, ** p < 0.01, *** p < 0.001 versus the control group.

Molecular docking was used to predict the binding sites between FC116 and microtubules. Molecular docking results demonstrated that FC116 could bind to Cys239 and Cys354 of the colchicine binding site of tubulin (PDB entry: 1SA0) (Fig. 3D and Fig. S5). Direct binding of FC116 to microtubulin was determined by MST assay with a K_d of 136 nM (Fig. 3E). To verify the important roles of Cys239 and Cys354 in the complex interaction, we prepared mutant microtubules by simultaneously inducing Cys239 and Cys354 point mutations, and further MST studies showed that FC116 lost its ability to bind to the mutant tubulin (Fig. 3F). These results suggested that FC116 bound to the colchicine binding site of the microtubules. Then colchicine was selected as a positive control for the microtubule inhibitory activity assay. The results showed that FC116 inhibited the polymerization of tubulin in a dose-dependent manner (Fig. 3G). This implied that FC116 was a

microtubule depolymerizing agent. Subsequently, the effect of FC116 on the microtubule networks was examined by immunofluorescence (Fig. 3H). The microtubule networks in control group showed normal organization. However, after treated with FC116 for 24 h, the microtubule networks were reduced and disorganized, indicating that the FC116 could inhibit the polymerization of microtubules at low concentrations. The above experiments demonstrated that FC116 directly bound to microtubules at colchicine binding sites and it was a microtubule depolymerizing agent.

2.5. FC116 induced endoplasmic reticulum (ER) stress

One of the most important roles of microtubules is to participate in mitosis, previous experimental results showed that FC116 could directly

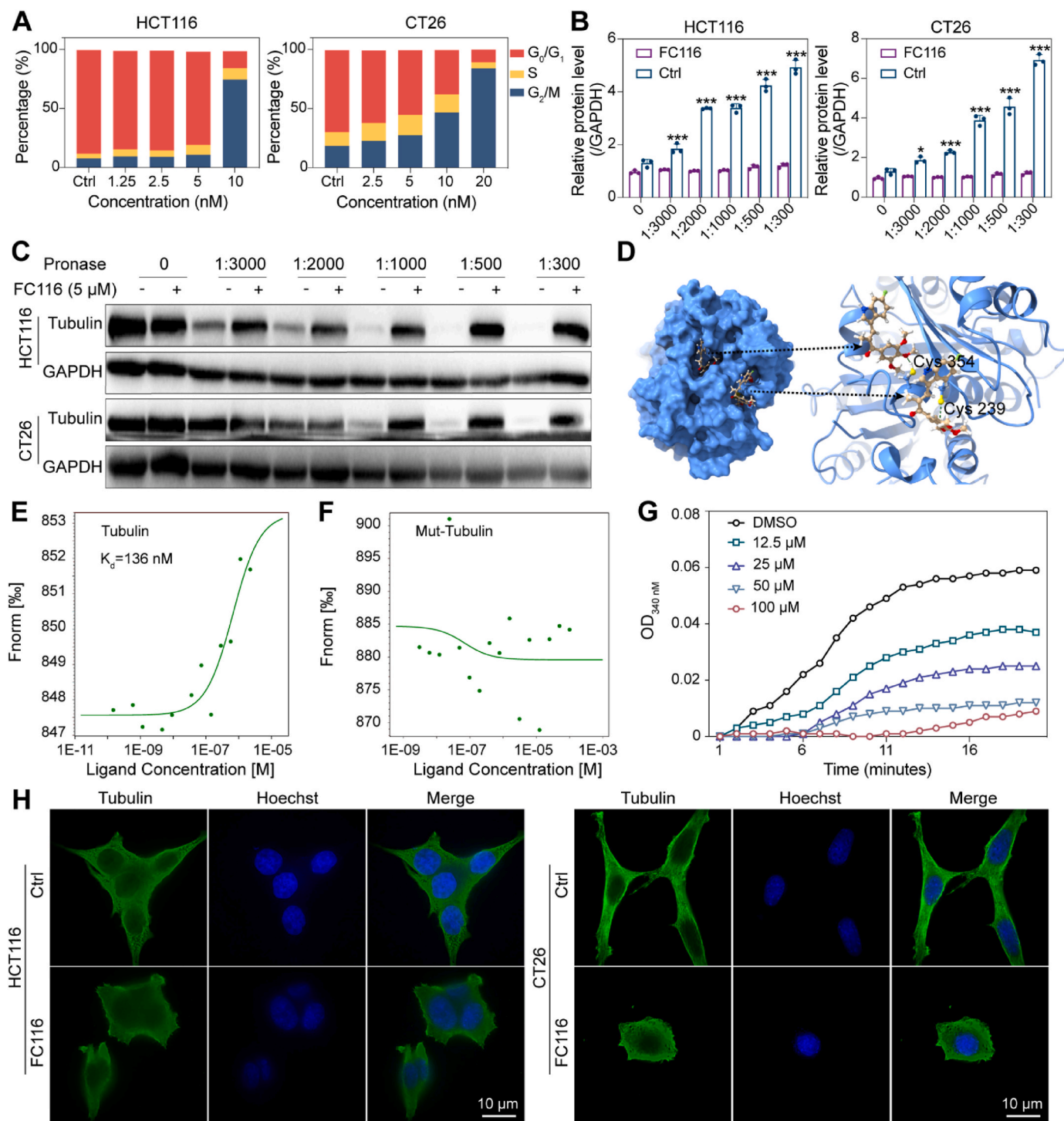


Fig. 3. FC116 directly bound to microtubules at colchicine binding sites. (A) Cell cycle changes of HCT116 and CT26 cells after FC116 administration were analyzed by flow cytometry. (B and C) The drug affinity reactive target stabilization (DARTS) assay was employed to detect an increase in tubulin after incubation of FC116 in pronase-digested HCT116 and CT26 cell lysates and immunoblot analysis of tubulin in pronase-digested cell lysate. (D) Molecular docking predicted the binding sites between FC116 and tubulin. (E) MST analysis of FC116 binding to wild-type tubulin ($K_d = 136$ nM). (F) MST analysis showed no binding of FC116 to mutant tubulin. (G) The polymerization of tubulin was monitored after treated with different concentrations of FC116. (H) Immunofluorescence detection of the microtubule networks of HCT116 and CT26 cells after treatment with FC116 by high-resolution microscopy. Data were shown as mean $\pm$ S.D. ($n = 3$), $*P < 0.05$, $***P < 0.001$ versus the control group.

bind to tubulin, inhibit their polymerization, induce apoptosis, and block the cell cycle. Inhibition of microtubule polymerization could impair the normal physiological function of ER, leading to excessive stress, producing overloaded ROS, impairing mitochondrial function, and inhibiting energy metabolism to induce cell apoptosis [39]. Here,

the results of transmission electron microscope (TEM) showed that after treated with FC116, more organelle vacuoles were visible in HCT116 and CT26 cells, and the rough ER was long-linear and irregularly dilated with locally verified expansion, indicating increased ER stress (Fig. 4A). The expression levels of ER stress-related proteins were detected by

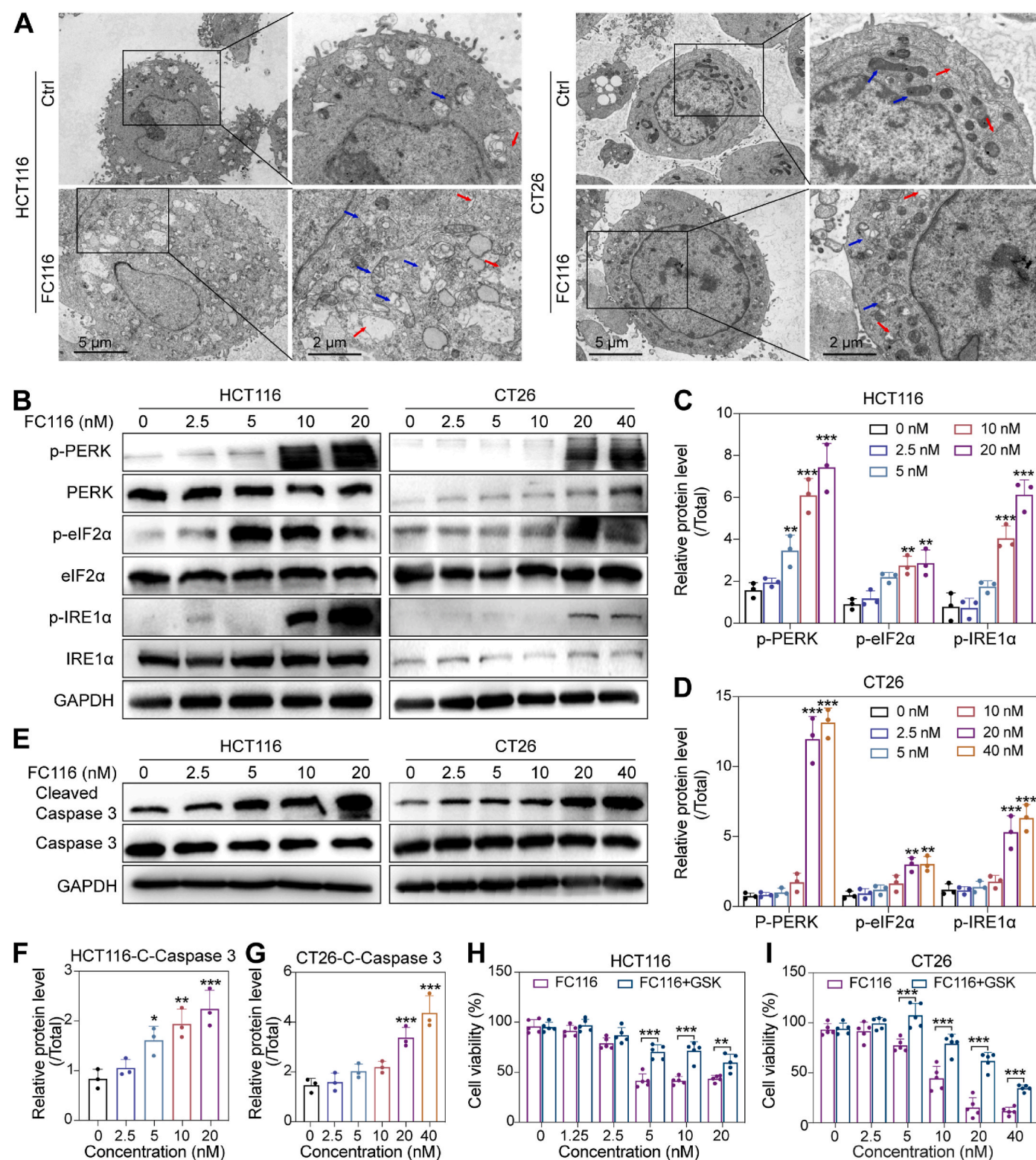


Fig. 4. FC116 induced ER stress. (A) Electron microscopic observation of organelle morphology in HCT116 and CT26 cells after 24 h incubation with FC116 (10 nM/20 nM); The red arrow points to ER, and the blue arrow indicates mitochondrial damage. (B) Western blotting assay to detect the expression levels of ER stress-related proteins after incubation with different concentrations of FC116 for 24 h on HCT116 and CT26 cells, and statistical assay of the relative protein contents (C–D). (E) The level of apoptosis-related proteins, Caspase 3 and Cleaved-Caspase 3 (C-Caspase3) on HCT116 and CT26 cells after treated with FC116 for 24 h and statistical analysis (F–G). (H–I) CCK-8 assay for cell proliferation after co-incubation of different concentrations of FC116 with GSK2606414 (2 μ M) for 24 h on CRC cell lines. Data were shown as mean $\pm$ S.D. (n = 3). * P < 0.05, ** P < 0.01, *** P < 0.001 versus the control group.

western blotting after FC116 treatment. The phosphorylation of PERK, eIF2 α , and IRE1 α is significantly increased, and the expression of apoptosis-related protein cleaved-caspase 3 was also up-regulated (Fig. 4B–G). To further verify the relationship between the inhibitory effects of FC116 and ER stress pathway, the PERK inhibitor, GSK2606414 (GSK), was added with FC116. GSK could block the ER stress pathway and significantly attenuate the of FC116 on CRC cells (Fig. 4H and I). These results showed that FC116 could stimulate the ER

stress-related p-PERK/p-eif2/p-IRE1 α axis to inhibit the growth of CRC cells.

2.6. FC116 impaired mitochondria and inhibited mitochondrial respiration

While observing the ER with TEM, we also found that mitochondria appeared significantly swollen, with increased volume, shallow and

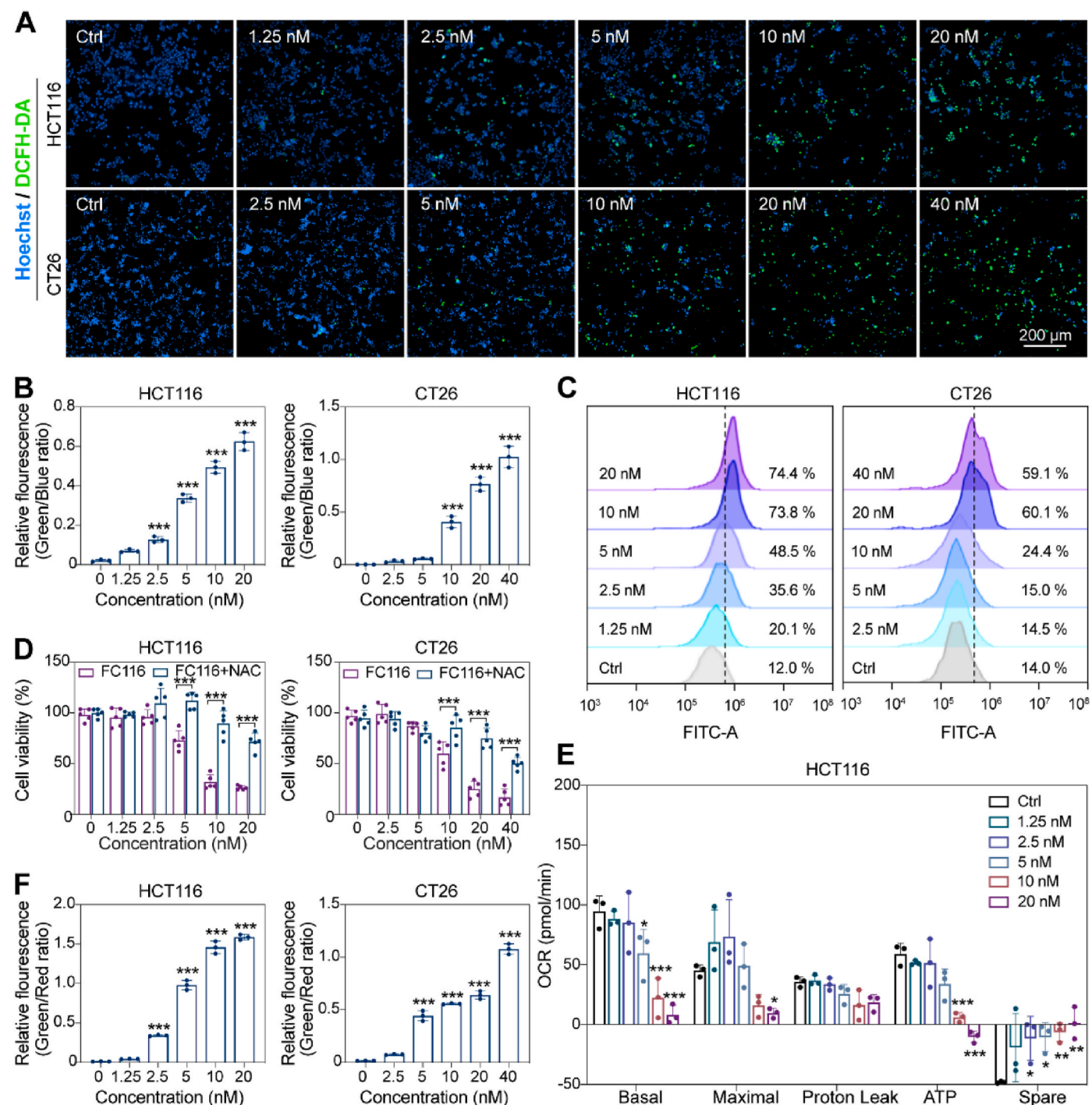


Fig. 5. FC116 impaired mitochondria and inhibited mitochondrial respiration. (A–C) DCFH-DA analysis showed that FC116 treatment significantly increased ROS levels on HCT116 and CT26 cells and statistical analysis. (D) CCK-8 assay for cell proliferation after co-incubation with different concentrations of FC116 and NAC (5 μ M) on HCT116 and CT26 cells. (E) Using JC-1 as a detector, FC116 treatment decreased MMP in HCT116 and CT26 cells. (F) Treatment with FC116 for 24 h significantly reduced the OCR of HCT116 cells in a dose-dependent manner. Data were shown as mean $\pm$ S.D. (n = 3), * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus the control group.

uneven matrix dissolution, massive crag fracture loss, and more mitochondrial vacuolization after treatment with FC116, indicating that mitochondrial damage occurred (Fig. 4A). MTAs have been reported inhibitory effect to increase the level of reactive oxygen species (ROS) via the mitochondrial pathway [40,41]. ROS is one of the major factors in ER stress [42,43]. Herein, the ROS level was increased in a dose-dependent manner after treated with FC116 both in HCT116 and CT26 cells (Fig. 5 A and B). The flow cytometric detection showed concentration-dependent induction of ROS production, with fluorescent signals increased to 74.4% and 59.1% in HCT116 and CT26 cells, respectively (Fig. 5C). The ER inhibitor GSK impeded the production of ROS induced by FC116 (Fig. S6). Then, the ROS scavenger, NAC, was used for combined treatment with FC116, which attenuated the

inhibition effect of FC116 on CRC cells (Fig. 5D). These results indicated that FC116 inhibited the growth of CRC cells via excess ROS induced by activating ER stress.

The decrease in mitochondrial membrane potential (MMP) is one of the main features of mitochondrial dysfunction. The change of MMP in CRC cells after treated with FC116 were determined by JC-1 fluorescence probe. The results revealed that FC116 could show a dose-dependent induction of mitochondrial dysfunction, manifested by a significant decrease in MMP (Fig. 5F and Fig. S7). As mitochondria plays an important role in cellular metabolism, it is reasonable to assume that FC116 would affect the energy metabolism of CRC cells. The oxygen consumption rate (OCR), one of the indicators of mitochondrial respiration detected by the Seahorse Bioscience XF96, was significantly

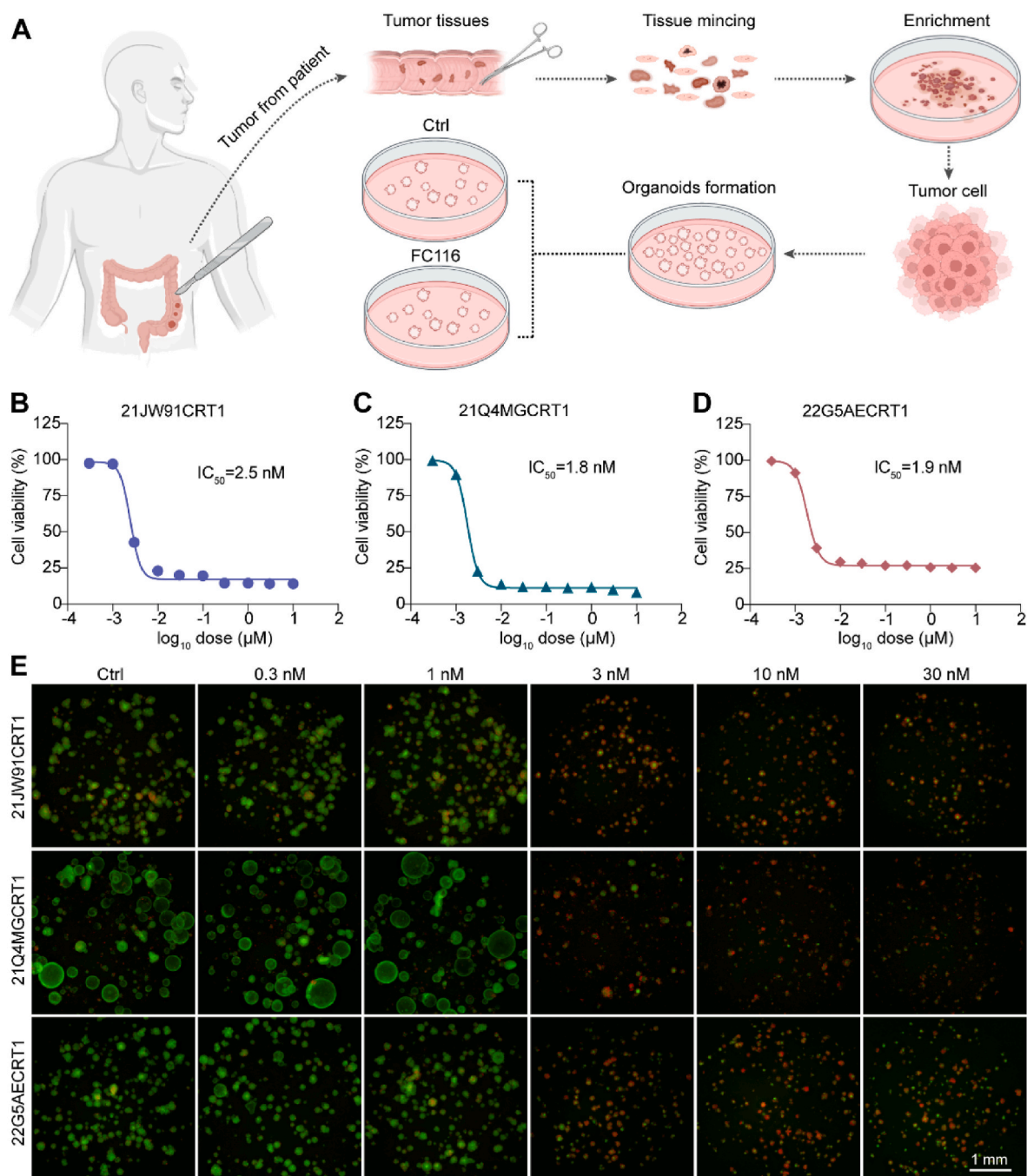


Fig. 6. FC116 inhibited the growth of CRC organoid models. (A) Schematic diagram of the construction and administration of patient-derived organs. (B–E) Drug sensitivity tests and representative immunofluorescence maps of organs derived from three patients.

reduced after treatment with FC116, indicating that mitochondrial respiration was inhibited (Fig. 5E).

2.7. FC116 inhibited the growth of CRC organoid models

Patient-derived organs (PDOs) are promising preclinical models with a high degree of consistency in composition and spatial structure, as well as in drug response to tissue, offering the possibility of becoming a tool for functional studies and drug screening, and providing a reference for individualized drug development. Therefore, three PDOs clinical model were used to further validate the antitumor efficacy of FC116 (Table S5). The organoids were firstly resuscitated by plating 100 organoids/well in a 48-well plate. After 3 days of incubation, FC116 was added to the wells when the organoids had grown to approximately 50–80 μm (Fig. 6A). Bright-field images of the organoids without treatment with FC116 were shown in the accompanying figure (Fig. S8). The results showed that the IC_{50} of FC116 on three different PDOs models was 2.06 ± 0.38 nM (Fig. 6B–D), which were consistent with the previous results *in vitro*. In addition, the fluorescent staining of live/dead organoids indicated the excellent inhibitory effects of FC116 (Fig. 6E).

2.8. FC116 reduced the occurrence of tumors in $\text{APC}^{\text{min}/+}$ mouse model

In addition to using the HCT116 xenograft tumor and PDOs models to verify the antitumor efficacy of FC116, a C57BL/6- $\text{APC}^{\text{min}/+}$ mouse model was constructed by replacing amino acid L at position 850 of the Apc gene with X (the termination mutation), which was regarded as an ideal mouse model for intestinal tumors.

[44]. Genotypic identification showed the successful construction of Apc-(L850X) mutant mice (Fig. S9). In the intestine of Apc-(L850X) mutant mice, the number of tumors increased gradually with the increase of weeks of age, and the mice were randomly divided into four groups at 10 weeks: control, FC116 (1.5 mg/kg/day), FC116 (3 mg/kg/day), and positive control PTX (7 mg/kg, three times every week) (Fig. 7A). After 45 days of FC116 treatment, there was no significant effect on the body weight of experimental mice. The numbers of tumor in the intestine were significantly reduced in the FC116 high-dose administration group compared to the control group (Fig. 7B–E and Fig. S10). H&E staining results showed no significant organ toxicity in FC116-treated mice (Fig. S11). Further immunohistochemical staining of xenograft tumors showed that the phosphorylation of eIF2 α and IRE1 α , the key proteins in the ER stress was increased significantly after treatment with FC116 (Fig. 7F–G), revealing that the ER stress was activated *in vivo* by FC116. All the results indicated that FC116 safely displayed potent antitumor effects *in vivo*.

3. Discussion

Microtubules are involved in the formation of the eukaryotic cytoskeleton and play key roles in cell division by forming the spindle during prophase mitosis, and abnormal cell division is a distinctive feature of cancer. Therefore, microtubules are regarded as important targets for the treatment of tumors. MTAs have been applied in the clinic and exerted preferable antitumor effects. However, the existing MTAs also have obvious limitations, such as poor biological stability and serious side effects, limiting their application [16,45]. Here, the SAR of the indole-chalcone compounds was explored to obtain the candidate compound FC116 with excellent antitumor effects on CRC. The lead compound FC77 was too toxic, and it could easily cause the death of mice. Therefore, we structurally modified it and obtained the fluorine-substituted compound FC116 at the indole 6 position after pharmacodynamic screening, which exerts promising antitumor efficacy *in vivo* without obvious toxicity. As a medicinal chemistry method, fluorine substitution is recognized to effectively influence the metabolic pathways and pharmacokinetic properties of molecules [39,46]. We speculate the introduction of fluorine also improve the biological safety

of FC116, and the specific reasons remain to be further elucidated.

In this experiment, FC116 could target microtubules on the colchicine binding site. FC116 was found to not only inhibit the proliferation, but also increase the expression of apoptosis-associated protein Cleaved-Caspase 3 and block the cell cycle in G₂/M phase, indicating that FC116 can target microtubules. Microtubules, the major components of cytoskeleton, are closely related to the function of ER [47]. Dissociating microtubules trigger the reorganization of ER and affect its function [48]. Among the wide variety of subcellular organelles, ER is the largest endosomal system in the cell, spanning the cytoplasm in a complex network capable of protein and lipid synthesis, protein modification, translocation, and secretion, which plays an important role in organelle communication [49]. Herein, we found that FC116 could increase the expression of ER-associated proteins *in vitro* and *in vivo*. Activation of ER stress could induce apoptosis, while the ER stress inhibitor (GSK) attenuated the inhibition effect of FC116 on CRC cells, revealing that FC116 inhibited the growth of CRC cells via activating ER stress.

The balance of mitochondrial fission and fusion with each other is critical to mitochondrial morphology and activity [50]. Microtubules act as channels for long-distance mitochondrial transport, providing the impetus for ER-mitochondrial contacts and keeping them physically tightly connected [48]. Microtubule instability could affect all microtubule-based processes, and interactions between the ER and mitochondria [51]. ROS is one of the major factors in ER stress. It was observed that the ROS was increased after treatment with FC116. Under physiological conditions, ROS regulates cell proliferation and survival, while excess ROS promotes genomic instability, interferes with normal cellular processes, and leads to cell death [52]. Here, the MMP, an index of mitochondria, was detected by the fluorescent probe JC-1 and a significant decrease was observed and the mitochondrial respiration was inhibited after FC116 treatment, suggesting that mitochondria were damaged. The combination of ROS scavenger (NAC) and FC116 significantly reversed the inhibition effects of FC116 on CRC cells. These results suggested that FC116 could destabilize microtubules, spoil the ER and mitochondrial function, leading to a series of physiological responses in cells to inhibit the growth of tumor cells.

Most of the existing microtubule-targeting inhibitors have low tumor selectivity, and could cause serious side effects. Herein, FC116 could inhibit the proliferation of various cancer cells, and the inhibitory effects on CRC cells are better than others (Table S6). It indicates that FC116 may possess other targets on CRC cell lines in addition to microtubules, so the selective targeting of FC116 to other targets in CRC needs to be further explored.

4. Conclusion

In this study, based on our previous work and the privileged structures of indole and chalcone, three new series of indole-chalcone derivatives were designed, and their SAR against CRC was explored. Among them, a representative fluorine-containing analog (FC16) was systematically investigated, and exhibited significant inhibitory activity toward HCT116 cells ($\text{IC}_{50} = 4.52$ nM), CT26 cells ($\text{IC}_{50} = 18.69$ nM), CRC organoids ($\text{IC}_{50} = 1.8\text{--}2.5$ nM), HCT116-xenograft mice (TGI value of 65.96% at the dose of 3 mg/kg), and $\text{APC}^{\text{min}/+}$ mice (adenoma number inhibition rate of 76.25% at the dose of 3 mg/kg). Meanwhile, the related mechanisms mediated by FC116 for CRC were also detailly studied. Our results support that indole-chalcone compounds are a class of promising lead MTAs and highlight the potential of FC116 to combat CRC.

5. Experimental section

5.1. Compounds and reagents

The obtained chemicals and solvents were not further purified, and all reagents were of market origin. NMR spectra were recorded on a

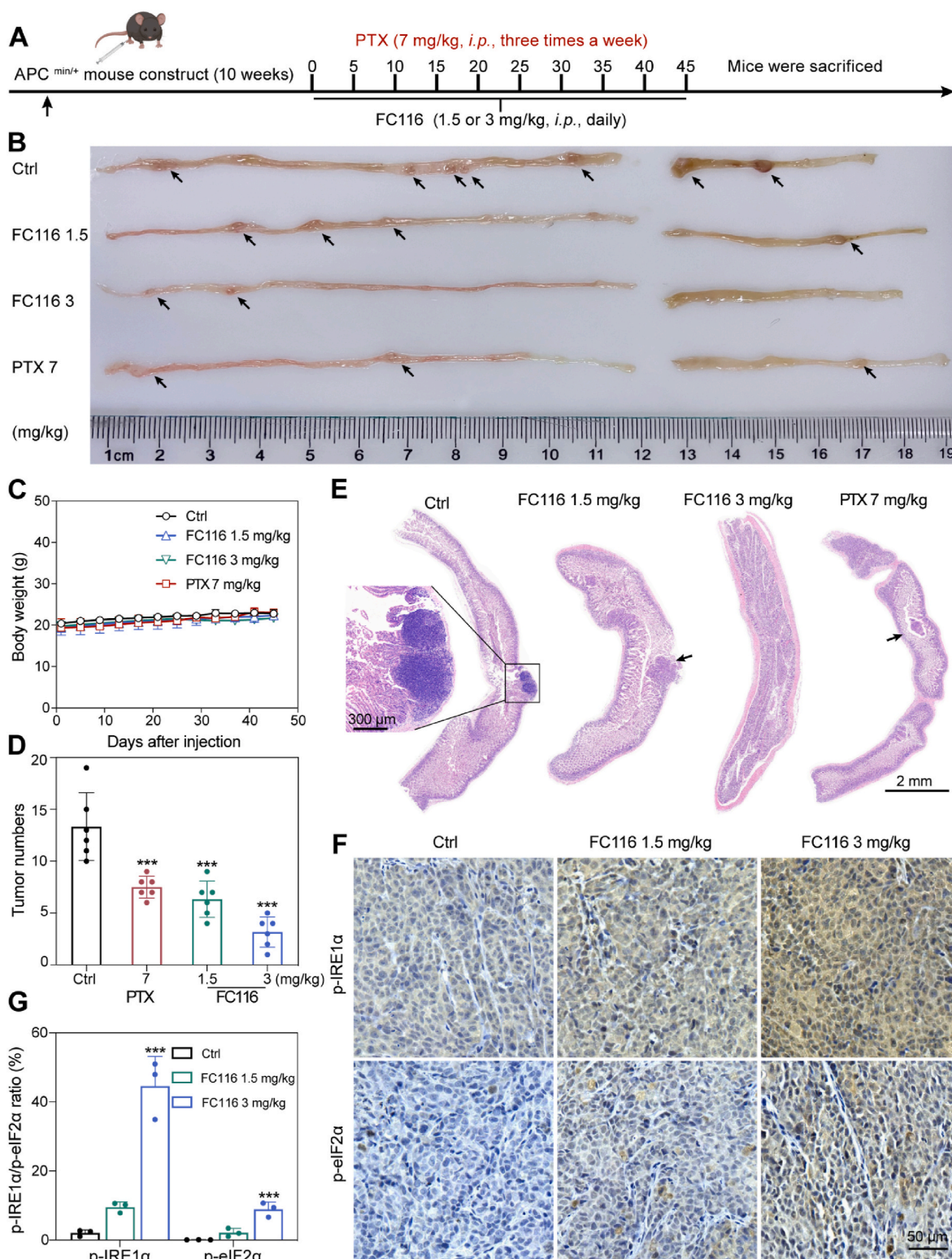


Fig. 7. FC116 reduced the occurrence of tumors in APC^{min/+} mouse model. (A) Schematic diagram of the planned flow of FC116 administration (1.5 and 3 mg/kg, *i.p.*, daily). (B) Representative picture of polyps in the intestinal region of mice. (C–D) The body weight of the mice was monitored once every two days for 45 days of continuous administration, and the number of intestinal tumors was counted after sacrificing the mice. Data were shown as mean $\pm$ S.D. ($n = 6$). * $P < 0.05$, *** $P < 0.001$ versus the control group. (E–F) Representative H&E staining images of transverse and longitudinal sections of intestinal polyps in mice. (F–G) In xenograft tumors, immunohistochemical detection of excised tumor P-eIF2 α and P-IRE1 α positive tumor cells was performed when mice were sacrificed. Data were shown as mean $\pm$ S.D. ($n = 3$). ** $P < 0.01$, *** $P < 0.001$ versus the control group.

Bruker AVANCE spectrometer (^1H , 300 MHz, 600 MHz; ^{13}C , 75 MHz, 150 MHz). Solvent peaks were used as reference values, with CDCl_3 at 7.26 ppm for ^1H NMR and 77.16 ppm for ^{13}C NMR, with $\text{DMSO}-d_6$ at 2.50 ppm for ^1H NMR and 39.52 ppm for ^{13}C NMR. Fourier transform infrared (FTIR) spectra were recorded on PerkinElmer Spectrum Two and then scanned from 600 to 4000 cm^{-1} at a resolution of 0.5 cm^{-1} . High-resolution mass spectrometry (HRMS) was performed by a Q-TOF micro mass spectrometer. The purities of the compounds were analyzed by HPLC (Agilent 1260) using a C18 column (Elite Hypersil ODS2, $4.6 \times 250\text{ mm}$, $5\text{ }\mu\text{m}$) with # mobile phase conditions (condition A: $\text{MeOH}/\text{H}_2\text{O}$ (100 vol%) for compounds **21**, **22**; condition B: $\text{MeOH}/\text{H}_2\text{O}$ (90/10 vol%) for compounds **14–17**, **25–28**; condition C: $\text{MeOH}/\text{H}_2\text{O}$ (80/20 vol%) for compounds **1–13**, **18–20**, **23**, **24**, **29–31**) at a flow rate of 2.0 mL/min . All final compounds exhibited purities greater than 95%.

FC116 was dissolved with 100% DMSO (Sigma, 01934-1L) to a final concentration of 20 mM , aliquots were dispensed and stored at 20°C . GAPDH (5174s, 1:1000), p-PERK (3700s, 1:1000), IRE1a (3294t, 1:1000), p-eIF2a (3398s, 1:1000), eIF2a (5324t, 1:1000), Cleaved-Caspase 3 (9664t, 1:1000), Caspase 3 (14220t, 1:1000), HRP-conjugated anti-rabbit IgG (1:5000), HRP-conjugated anti-mouse IgG (1:5000) antibody were all purchased from Cell Signaling Technology (CST, Danvers, MA, USA). The antibody for p-PERK (ap0886, 1:1000) was purchased from ABClonal Technology Co., Ltd. (Wuhan, China). The antibody for p-IRE1a (bs-16698r, 1:1000) was purchased from Bioss (Beijing, China). The antibody for β -Tubulin (abs-830032, 1:1000) was purchased from Absin (Shanghai, China). GSK2606414 (t2614) and N-acetylcysteine (NAC) (t0875) were obtained from Topscience (Shanghai, China).

5.1.1. (E)-3-(1H-indol-3-yl)-2-methyl-1-phenylprop-2-en-1-one (1)

To a solution of propiophenone (201.3 mg , 1.5 mmol) in toluene (6 mL) was added indole-3-carboxaldehyde (72.6 mg , 0.5 mmol) and piperidine (0.3 mL). After stirred at 110°C for 4 h , the reaction mixture was filtered and the solid was recrystallized in ethanol at -20°C for 24 h to obtain compound with a yield of 57%. m.p. $207.1\text{--}207.9^\circ\text{C}$. ^1H NMR (600 MHz , CDCl_3) δ 68.76 (s, 1H), 7.73–7.75 (m, 2H), 7.62–7.64 (m, 2H), 7.55–7.5 (m, 1H), 7.47–7.52 (m, 3H), 7.42 (d, 1H, $J = 7.8\text{ Hz}$), 7.25–7.28 (m, 1H), 7.15–7.18 (m, 1H), 2.33 (s, 3H). ^{13}C NMR (75 MHz , CDCl_3) δ 199.23, 139.65, 135.54, 132.15, 131.18, 129.46 (2C), 128.24 (2C), 127.69, 126.21, 123.47, 121.06, 118.69, 113.50, 111.56, 15.20. HRMS: $[\text{M}+\text{H}]^+ m/z$ calcd for $\text{C}_{18}\text{H}_{16}\text{NO}^+$: 262.1232, found: 262.1232. HPLC analysis: retention time = 8.7 min ; peak area, >95% (254 nm).

5.1.2. (E)-3-(1H-indol-3-yl)-1-phenylprop-2-en-1-one (2)

To a solution of acetophenone (123.7 mg , 1.03 mmol) in ethanol (5 mL) was added indole-3-carboxaldehyde (49.82 mg , 0.34 mmol) and piperidine (0.2 mL). After stirred at 95°C for 48 h , the mixture was concentrated and recrystallized in ethanol at -20°C for 24 h to obtain compound with a yield of 55%. m.p. $165.9\text{--}167.5^\circ\text{C}$. ^1H NMR (400 MHz , CDCl_3) δ 9.03 (s, 1H), 7.76 (d, $J = 7.8\text{ Hz}$, 2H), 7.66 (d, $J = 6.7\text{ Hz}$, 2H), 7.61–7.47 (m, 4H), 7.45 (d, $J = 8.1\text{ Hz}$, 1H), 7.29 (d, $J = 5.5\text{ Hz}$, 1H), 7.18 (t, $J = 7.5\text{ Hz}$, 1H), 2.35 (s, 3H). ^{13}C NMR (101 MHz , CDCl_3) δ 199.10, 139.59, 135.51, 131.92, 131.01, 129.31 (2C), 128.10 (3C), 127.60, 126.20, 123.26, 120.88, 118.53, 113.29, 111.48, 15.04. HRMS: $[\text{M}+\text{H}]^+ m/z$ calcd for $\text{C}_{17}\text{H}_{14}\text{NO}^+$: 248.1075, found: 248.1077. HPLC analysis: retention time = 31.2 min ; peak area, >95% (254 nm).

5.1.3. (E)-1-(3,5-dimethoxyphenyl)-3-(1H-indol-3-yl)-2-methylprop-2-en-1-one (3)

Compound **3** was prepared following a similar procedure to compound **2** with a yield of 51%. ^1H NMR (300 MHz , CDCl_3) δ 8.73 (s, 1H), 7.70 (s, 1H), 7.63 (s, 1H), 7.56 (d, 1H, $J = 7.8\text{ Hz}$), 7.43 (d, 1H, d_6 $J = 7.8\text{ Hz}$), 7.16–7.21 (m, 1H), 6.86 (s, 3H), 6.64 (s, 1H), 3.83 (s, 6H), 2.31 (s, 3H). ^{13}C NMR (150 MHz , $\text{DMSO}-d_6$) δ 198.72, 160.54, 141.64, 135.61, 135.49, 131.93, 127.72, 126.29, 123.48, 121.12, 118.75, 113.51, 111.56, 107.18, 103.69, 55.71, 15.19. HRMS: $[\text{M}+\text{H}]^+ m/z$

calcd for $\text{C}_{20}\text{H}_{20}\text{NO}_3^+$: 322.1443, found: 322.1444. HPLC analysis: retention time = 9.2 min ; peak area, >95% (254 nm).

5.1.4. (E)-1-(3,4-dimethoxyphenyl)-3-(1H-indol-3-yl)-2-methylprop-2-en-1-one (4)

Compound **4** was prepared following a similar procedure to compound **2** with a yield of 52%. m.p. $170.0\text{--}170.3^\circ\text{C}$. ^1H NMR (300 MHz , CDCl_3) δ 8.72 (s, 1H), 7.57–7.62 (m, 3H), 7.40–7.45 (m, 3H), 7.30 (s, 1H), 7.16–7.21 (m, 1H), 6.93 (d, 1H, $J = 8.1\text{ Hz}$), 3.96 (d, 6H, $J = 13.2\text{ Hz}$), 2.32 (s, 3H). ^{13}C NMR (150 MHz , $\text{DMSO}-d_6$) δ 198.15, 152.04, 148.86, 135.61, 133.68, 132.18, 131.90, 127.75, 125.89, 124.03, 123.41, 121.00, 118.71, 134.48, 112.41, 111.55, 109.91, 56.14, 15.83. HRMS: $[\text{M}+\text{H}]^+ m/z$ calcd for $\text{C}_{20}\text{H}_{20}\text{NO}_3^+$: 322.1443, found: 322.1447. HPLC analysis: retention time = 7.0 min ; peak area, >95% (254 nm).

5.1.5. (E)-3-(1H-indol-3-yl)-1-(3-methoxyphenyl)-2-methylprop-2-en-1-one (5)

Compound **5** was prepared following a similar procedure to compound **2** with a yield of 46%. m.p. $129.6\text{--}130.2^\circ\text{C}$. ^1H NMR (300 MHz , CDCl_3) δ 8.72 (s, 1H), 7.65 (d, 2H, $J = 12.3\text{ Hz}$), 7.53 (d, 1H, $J = 7.8\text{ Hz}$), 7.36–7.44 (m, 2H), 7.26–7.31 (m, 3H), 7.08–7.20 (m, 2H), 3.86 (s, 3H), 2.31 (s, 3H). ^{13}C NMR (150 MHz , $\text{DMSO}-d_6$) δ 198.92, 159.58, 141.00, 135.62, 135.49, 132.07, 129.13, 127.71, 126.25, 123.48, 122.02, 121.09, 118.72, 117.58, 113.98, 113.51, 111.56, 55.56, 15.21. HRMS: $[\text{M}+\text{H}]^+ m/z$ calcd for $\text{C}_{19}\text{H}_{18}\text{NO}_2^+$: 292.1338, found: 292.1340. HPLC analysis: retention time = 8.8 min ; peak area, >95% (254 nm).

5.1.6. (E)-3-(1H-indol-3-yl)-1-(7-methoxybenzo[d][1,3]dioxol-5-yl)-2-methylprop-2-en-1-one (6)

Compound **6** was prepared following a similar procedure to compound **2** with a yield of 51%. m.p. $198.6\text{--}199.3^\circ\text{C}$. ^1H NMR (300 MHz , CDCl_3) δ 8.66 (s, 1H), 7.59–7.62 (m, 3H), 7.44 (d, 1H, $J = 7.8\text{ Hz}$), 7.28–7.31 (m, 1H), 7.17–7.22 (m, 1H), 7.06 (s, 1H), 6.99 (s, 1H), 6.09 (s, 2H), 3.93 (s, 3H), 2.30 (s, 3H). ^{13}C NMR (150 MHz , $\text{DMSO}-d_6$) δ 197.55, 148.55, 143.40, 135.60, 133.83, 132.03, 127.71, 126.00, 123.47, 121.09, 118.73, 113.44, 111.55, 110.03, 104.46, 102.30, 56.74, 15.79. HRMS: $[\text{M}+\text{H}]^+ m/z$ calcd for $\text{C}_{20}\text{H}_{18}\text{NO}_4^+$: 336.1236, found: 336.1237. HPLC analysis: retention time = 5.2 min ; peak area, >95% (254 nm).

5.1.7. (E)-3-(1H-indol-3-yl)-1-(5-methoxypyridin-3-yl)-2-methylprop-2-en-1-one (7)

Compound **7** was prepared following a similar procedure to compound **2** with a yield of 46%. m.p. $143.0\text{--}143.5^\circ\text{C}$. ^1H NMR (300 MHz , CDCl_3) δ 8.93 (s, 1H), 8.51 (d, 2H, $J = 18.0\text{ Hz}$), 7.67 (s, 2H), 7.53 (d, 2H, $J = 9.0\text{ Hz}$), 7.44 (d, 1H, $J = 9.0\text{ Hz}$), 7.30 (s, 1H), 7.15–7.20 (m, 1H), 3.93 (s, 3H), 2.32 (s, 3H). ^{13}C NMR (150 MHz , $\text{DMSO}-d_6$) δ 96.32, 159.31, 142.06, 140.38, 136.58, 135.94, 135.66, 131.99, 127.59, 126.78, 123.67, 121.35, 120.44, 118.66, 113.34, 111.64, 55.91, 14.97. HRMS: $[\text{M}+\text{H}]^+ m/z$ calcd for $\text{C}_{18}\text{H}_{17}\text{N}_2\text{O}_2^+$: 293.1290, found: 293.1290. HPLC analysis: retention time = 6.7 min ; peak area, >95% (254 nm).

5.1.8. (E)-2-((1H-indol-3-yl)methylene)-1-(3,4,5-trimethoxyphenyl)butan-1-one (8)

Compound **8** was prepared following a similar procedure to compound **2** with a yield of 43%. ^1H NMR (400 MHz , CDCl_3) δ 9.13 (s, 1H), 7.64 (d, $J = 13.8\text{ Hz}$, 2H), 7.57 (d, $J = 7.9\text{ Hz}$, 1H), 7.45 (d, $J = 8.1\text{ Hz}$, 1H), 7.29 (d, $J = 7.1\text{ Hz}$, 1H), 7.21 (dd, $J = 14.3$, 6.7 Hz , 1H), 7.07 (s, 2H), 3.99 (s, 3H), 3.91 (s, 6H), 2.87 (d, $J = 7.5\text{ Hz}$, 2H), 1.31 (d, $J = 7.2\text{ Hz}$, 3H). ^{13}C NMR (101 MHz , CDCl_3) δ 198.17, 152.83 (2C), 140.86, 137.90, 135.66, 134.92, 133.84, 127.81, 125.40, 123.22, 120.98, 118.33, 112.10, 111.61, 107.02 (2C), 61.01, 56.30 (2C), 21.96, 12.31. HRMS: $[\text{M}+\text{H}]^+ m/z$ calcd for $\text{C}_{22}\text{H}_{24}\text{NO}_4^+$: 366.1705, found: 366.1706.

5.1.9. (E)-2-((1H-indol-3-yl)methylene)-5-methoxy-3,4-dihydronaphthalen-1(2H)-one (9)

Compound **9** was prepared following a similar procedure to

compound **2** with a yield of 52%. ^1H NMR (400 MHz, CDCl_3) δ 8.69 (s, 1H), 8.24 (s, 1H), 7.88 (d, $J = 7.7$ Hz, 1H), 7.78 (d, $J = 7.9$ Hz, 1H), 7.55 (d, $J = 2.3$ Hz, 1H), 7.43 (d, $J = 7.8$ Hz, 1H), 7.31 (dd, $J = 14.8, 7.3$ Hz, 2H), 7.23 (d, $J = 7.3$ Hz, 1H), 7.04 (d, $J = 8.1$ Hz, 1H), 3.88 (s, 3H), 3.13 (t, $J = 6.7$ Hz, 2H), 2.99 (t, $J = 6.6$ Hz, 2H). ^{13}C NMR (101 MHz, CDCl_3) δ 156.23, 135.71, 135.09, 131.85, 130.92, 128.46, 128.04, 127.03, 125.69, 123.27, 121.00, 119.90, 119.46, 113.98, 113.50, 111.32, 55.76, 27.42, 20.85. HRMS: $[\text{M}+\text{H}]^+$ m/z calcd for $\text{C}_{20}\text{H}_{18}\text{NO}_2^+$: 304.1338, found: 304.1340. HPLC analysis: retention time = 9.3 min; peak area, >95% (254 nm).

5.1.10. (*E*)-2-methyl-3-(4-methyl-1*H*-indol-3-yl)-1-(3,4,5-trimethoxyphenyl)prop-2-en-1-one (10)

Compound **10** was prepared following a similar procedure to compound **1** with a yield of 51%. m.p. 213.5–214.4 °C. ^1H NMR (300 MHz, CDCl_3) δ 8.69 (s, 1H), 7.97 (s, 1H), 7.60 (d, 1H, $J = 1.2$ Hz), 7.26–7.29 (m, 1H), 7.10–7.16 (m, 1H), 6.89–6.95 (m, 3H), 3.89–3.91 (m, 9H), 2.47 (s, 3H), 2.29 (s, 3H). ^{13}C NMR (150 MHz, $\text{DMSO}-d_6$) δ 198.91, 152.95, 140.51, 138.45, 136.06, 135.23, 131.92, 131.01, 126.85, 125.83, 123.34, 114.47, 109.69, 106.46, 61.12, 56.39, 21.36, 14.99. HRMS: $[\text{M}+\text{H}]^+$ m/z calcd for $\text{C}_{22}\text{H}_{24}\text{NO}_4^+$: 366.1705, found: 366.1702. HPLC analysis: retention time = 38.6 min; peak area, >95% (254 nm).

5.1.11. (*E*)-2-methyl-3-(5-methyl-1*H*-indol-3-yl)-1-(3,4,5-trimethoxyphenyl)prop-2-en-1-one (11)

Compound **11** was prepared following a similar procedure to compound **1** with a yield of 50%. m.p. 192.9–193.3 °C. ^1H NMR (600 MHz, CDCl_3) δ 8.55 (s, 1H), 7.66 (s, 1H), 7.60 (d, 1H, $J = 1.6$ Hz), 7.32–7.35 (m, 2H), 7.10 (d, 1H, $J = 1.8$ Hz), 7.03 (s, 2H), 3.96 (s, 3H), 3.91 (s, 6H), 2.44 (s, 3H), 2.31 (s, 3H). ^{13}C NMR (600 MHz, $\text{DMSO}-d_6$) δ 198.13, 152.92, 140.79, 134.86, 133.90, 131.54, 130.71, 128.03, 126.29, 125.12, 118.22, 113.02, 111.26, 107.07, 61.15, 56.39, 21.07, 15.56. HRMS: $[\text{M}+\text{H}]^+$ m/z calcd for $\text{C}_{22}\text{H}_{24}\text{NO}_4^+$: 366.1705, found: 366.1702. HPLC analysis: retention time = 6.7 min; peak area, >95% (254 nm).

5.1.12. (*E*)-2-methyl-3-(6-methyl-1*H*-indol-3-yl)-1-(3,4,5-trimethoxyphenyl)prop-2-en-1-one (12)

Compound **12** was prepared following a similar procedure to compound **1** with a yield of 46%. m.p. 174.0–174.4 °C. ^1H NMR (600 MHz, CDCl_3) δ 8.54 (s, 1H), 7.67 (s, 1H), 7.60 (d, 1H, $J = 3.0$ Hz), 7.32–7.34 (m, 2H), 7.10 (d, 1H, $J = 8.4$ Hz), 7.03 (s, 2H), 3.96 (s, 3H), 3.90 (s, 6H), 2.44 (s, 3H), 2.31 (d, 3H, $J = 1.2$ Hz). ^{13}C NMR (150 MHz, $\text{DMSO}-d_6$) δ 198.13, 152.92, 136.06, 134.91, 133.52, 131.65, 125.63, 122.95, 118.23, 113.37, 111.50, 107.10, 61.13, 56.41, 21.81, 15.51. HRMS: $[\text{M}+\text{H}]^+$ m/z calcd for $\text{C}_{22}\text{H}_{24}\text{NO}_4^+$: 366.1705, found: 366.1713. HPLC analysis: retention time = 8.3 min; peak area, >95% (254 nm).

5.1.13. (*E*)-2-methyl-3-(7-methyl-1*H*-indol-3-yl)-1-(3,4,5-trimethoxyphenyl)prop-2-en-1-one (13)

Compound **13** was prepared following a similar procedure to compound **1** with a yield of 48%. m.p. 169.9–170.6 °C. ^1H NMR (300 MHz, CDCl_3) δ 8.57 (s, 1H), 7.66 (d, 2H, $J = 10.2$ Hz), 7.43 (d, 1H, $J = 7.2$ Hz), 7.10–7.15 (m, 2H), 7.02 (s, 2H), 3.89–3.95 (m, 9H), 2.54 (s, 3H), 2.32 (s, 3H). HRMS: $[\text{M}+\text{H}]^+$ m/z calcd for $\text{C}_{22}\text{H}_{24}\text{NO}_4^+$: 366.1705, found: 366.1706. HPLC analysis: retention time = 8.5 min; peak area, >95% (254 nm).

5.1.14. (*E*)-3-(5-fluoro-1*H*-indol-3-yl)-2-methyl-1-(3,4,5-trimethoxyphenyl)prop-2-en-1-one (14)

Compound **14** was prepared following a similar procedure to compound **2** with a yield of 51%. m.p. 227.5–229.3 °C. ^1H NMR (300 MHz, CDCl_3) δ 8.62 (s, 1H), 7.67 (s, 1H), 7.54 (s, 1H), 7.36 (dd, 1H, $J = 4.5, 8.7$ Hz), 7.20–7.26 (m, 1H), 7.02 (s, 3H), 3.90–3.96 (m, 9H), 2.31 (s, 3H). ^{13}C NMR (150 MHz, $\text{DMSO}-d_6$) δ 197.99, 159.57, 153.02, 141.20, 134.41, 133.63, 132.37, 132.09, 127.43, 113.69, 112.43, 112.37, 112.12, 111.94, 107.21, 104.01, 103.88, 61.15, 56.47, 15.64. HRMS:

$[\text{M}+\text{H}]^+$ m/z calcd for $\text{C}_{21}\text{H}_{21}\text{FNO}_4^+$: 370.1455, found: 370.1454. HPLC analysis: retention time = 7.3 min; peak area, >95% (254 nm).

5.1.15. (*E*)-3-(5-chloro-1*H*-indol-3-yl)-2-methyl-1-(3,4,5-trimethoxyphenyl)prop-2-en-1-one (15)

Compound **15** was prepared following a similar procedure to compound **2** with a yield of 57%. m.p. 171.2–173.7 °C. ^1H NMR (300 MHz, CDCl_3) δ 10.04 (s, 1H), 7.60 (d, 2H, $J = 11.7$ Hz), 7.44–7.49 (m, 2H), 7.01 (s, 3H), 3.89–3.96 (m, 9H), 2.31 (s, 3H). ^{13}C NMR (150 MHz, $\text{DMSO}-d_6$) δ 198.18, 152.95, 140.97, 136.00, 134.44, 133.87, 132.52, 129.39, 126.57, 126.33, 123.85, 123.05, 121.87, 119.52, 113.42, 111.61, 107.05, 61.15, 56.41, 15.61. HRMS: $[\text{M}+\text{H}]^+$ m/z calcd for $\text{C}_{21}\text{H}_{21}\text{ClNO}_4^+$: 386.1159, found: 386.1157. HPLC analysis: retention time = 10.2 min; peak area, >95% (254 nm).

5.1.16. (*E*)-3-(5-bromo-1*H*-indol-3-yl)-2-methyl-1-(3,4,5-trimethoxyphenyl)prop-2-en-1-one (16)

Compound **16** was prepared following a similar procedure to compound **1** with a yield of 54%. ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ 12.07 (s, 1H), 7.94 (s, 1H), 7.67 (s, 1H), 7.56 (s, 1H), 7.46 (d, $J = 8.6$ Hz, 1H), 7.31 (d, $J = 8.6$ Hz, 1H), 7.00 (s, 2H), 3.83 (s, 6H), 3.79 (s, 3H), 2.22 (s, 3H). ^{13}C NMR (101 MHz, $\text{DMSO}-d_6$) δ 196.97, 152.95 (2C), 140.72, 135.02, 134.62, 134.05, 130.95, 129.72, 125.40, 120.73, 114.59, 113.35, 111.43, 107.33 (2C), 60.65, 56.50 (2C), 15.67.

5.1.17. (*E*)-3-(6-fluoro-1*H*-indol-3-yl)-2-methyl-1-(3,4,5-trimethoxyphenyl)prop-2-en-1-one (17)

Compound **17** was prepared following a similar procedure to compound **2** with a yield of 55%. m.p. 200–202.2 °C. ^1H NMR (300 MHz, CDCl_3) δ 8.85 (s, 1H), 7.60 (s, 2H), 7.46–7.50 (m, 1H), 7.11 (d, 1H, $J = 9.3$ Hz), 6.92–7.02 (m, 3H), 3.89–3.95 (m, 9H), 2.30 (s, 3H). ^{13}C NMR (150 MHz, $\text{DMSO}-d_6$) δ 198.19, 152.94, 140.93, 135.70, 134.52, 134.11, 132.42, 126.37, 124.24, 119.54, 119.40, 113.38, 110.09, 107.04, 98.21, 97.86, 61.15, 56.41, 15.57. IR (ATR) ν : 3319.26, 2940.53, 1635.08, 1612.84, 1579.03, 1516.94, 1503.66, 1453.68, 1408.78, 1350.38, 1328.73, 1316.82, 1304.60, 1264.35, 1248.46, 1229.99, 1180.38, 1122.14, 1075.08, 1049.87, 1022.58, 997.38, 952.32, 862.51, 853.52, 835.03, 808.12, 770.88, 751.71, 683.94, 666.94 cm^{-1} . HRMS: $[\text{M}+\text{H}]^+$ m/z calcd for $\text{C}_{21}\text{H}_{21}\text{FNO}_4^+$: 370.1455, found: 370.1450. HPLC analysis: retention time = 5.6 min; peak area, >95% (254 nm).

5.1.18. (*E*)-2-methyl-3-(5-nitro-1*H*-indol-3-yl)-1-(3,4,5-trimethoxyphenyl)prop-2-en-1-one (18)

Compound **18** was prepared following a similar procedure to compound **2** with a yield of 41%. ^1H NMR (300 MHz, $\text{DMSO}-d_6$) δ 12.50 (s, 1H), 8.49 (s, 1H), 8.07–8.13 (d, 2H), 7.65 (d, 2H, $J = 9.0$ Hz), 7.04 (s, 2H), 3.78–3.84 (m, 9H), 2.23 (s, 3H). ^{13}C NMR (150 MHz, $\text{DMSO}-d_6$) δ 162.66, 159.45, 156.50, 149.49, 146.64, 138.11, 137.52, 134.69, 133.86, 128.68, 128.47, 128.24, 123.98, 121.33, 120.19, 117.82, 116.06, 114.97, 74.44, 67.77, 46.02. HRMS: $[\text{M}+\text{H}]^+$ m/z calcd for $\text{C}_{21}\text{H}_{21}\text{N}_2\text{O}_6^+$: 397.1400, found: 397.1400. HPLC analysis: retention time = 7.9 min; peak area, >95% (254 nm).

5.1.19. (*E*)-2-methyl-3-(6-nitro-1*H*-indol-3-yl)-1-(3,4,5-trimethoxyphenyl)prop-2-en-1-one (19)

Compound **19** was prepared following a similar procedure to compound **2** with a yield of 43%. m.p. 267.0–269.3 °C. ^1H NMR (300 MHz, $\text{DMSO}-d_6$) δ 12.55 (s, 1H), 8.35 (d, 2H, $J = 27.3$ Hz), 7.98 (d, 1H, $J = 8.7$ Hz), 7.71 (d, 1H, $J = 8.7$ Hz), 7.57 (s, 1H), 6.99 (s, 2H), 3.78–3.81 (m, 9H), 2.24 (s, 3H). ^{13}C NMR (150 MHz, $\text{DMSO}-d_6$) δ 196.59, 152.51, 142.81, 140.31, 134.34, 133.83, 132.52, 131.91, 131.74, 118.50, 115.33, 112.02, 108.79, 106.74, 60.20, 56.06, 15.28. HRMS: $[\text{M}+\text{H}]^+$ m/z calcd for $\text{C}_{21}\text{H}_{19}\text{N}_2\text{O}_6^+$: 395.1242, found: 395.1269. HPLC analysis: retention time = 30.7 min; peak area, >95% (254 nm).

5.1.20. (E)-2-methyl-3-(7-nitro-1H-indol-3-yl)-1-(3,4,5-trimethoxyphenyl)prop-2-en-1-one (20)

Compound **20** was prepared following a similar procedure to compound **2** with a yield of 39%. m.p. 210.0–211.6 °C. ¹H NMR (300 MHz, DMSO-*d*₆) δ 12.55 (s, 1H), 8.20 (d, 1H, *J* = 27.3 Hz), 8.07 (d, 1H, *J* = 8.7 Hz), 7.91 (d, 1H, *J* = 8.7 Hz), 7.57 (s, 1H), 7.34 (t, 1H, *J* = 7.8 Hz), 7.01 (s, 2H), 3.78–3.81 (m, 9H), 2.25 (s, 3H). HRMS: [M+H]⁺ *m/z* calcd for C₂₁H₂₁N₂O₆⁺: 397.1400, found: 397.1400. HPLC analysis: retention time = 7.7 min; peak area, >95% (254 nm).

5.1.21. (E)-3-(6-amino-1H-indol-3-yl)-2-methyl-1-(3,4,5-trimethoxyphenyl)prop-2-en-1-one (21)

To a solution of (E)-2-methyl-3-(6-nitro-1H-indol-3-yl)-1-(3,4,5-trimethoxyphenyl)prop-2-en-1-one (0.3 mmol, 118.8 mg) in ethanol/water (5:1), added NH₄Cl (2.1 mmol, 112 mg) and iron powder (117.6 mg, 2.1 mmol), then warmed up to 55 °C for 6 h. The reaction mixture was filtered through a pad of celite and concentrated under reduced pressure, then recrystallized in ethanol at –20 °C for 24 h to obtain compound **21** with a yield of 82%. ¹H NMR (300 MHz, CDCl₃) δ 11.35 (s, 1H), 7.54 (s, 2H), 7.08 (d, 1H, *J* = 3.0 Hz), 6.92 (s, 2H), 6.60 (d, 2H, *J* = 3.0 Hz), 6.47–6.48 (m, 1H), 4.89 (s, 1H), 3.77–3.80 (m, 9H), 2.16 (s, 3H). HRMS: [M+H]⁺ *m/z* calcd for C₂₁H₂₃N₂O₄⁺: 367.1658, found: 367.1656.

5.1.22. (E)-3-(6-methoxy-1H-indol-3-yl)-2-methyl-1-(3,4,5-trimethoxyphenyl)prop-2-en-1-one (22)

Compound **22** was prepared following a similar procedure to compound **2** with a yield of 63%. ¹H NMR (600 MHz, CDCl₃) δ 8.47 (s, 1H), 7.62 (s, 1H), 7.52 (d, 1H, *J* = 2.4 Hz), 7.45 (s, 1H), 7.43 (s, 1H), 7.02 (s, 1H), 6.91 (d, 1H, *J* = 2.4 Hz), 6.85–6.86 (dd, 1H, *J* = 2.4, 9.0 Hz), 3.95 (s, 3H), 3.89 (s, 6H), 3.86 (s, 3H), 2.30 (s, 3H). ¹³C NMR (150 MHz, DMSO-*d*₆) δ 198.54, 157.51, 152.93, 146.21, 136.42, 134.71, 131.96, 125.01, 122.84, 119.28, 113.51, 111.08, 107.09, 94.94, 61.14, 56.43, 55.86, 15.51. HRMS: [M+H]⁺ *m/z* calcd for C₂₂H₂₄NO₅⁺: 382.1654, found: 382.1659. HPLC analysis: retention time = 19.9 min; peak area, >95% (254 nm).

5.1.23. methyl(E)-3-(2-methyl-3-oxo-3-(3,4,5-trimethoxyphenyl)prop-1-en-1-yl)-1H-indole-6-carboxylate (23)

Compound **23** was prepared following a similar procedure to compound **2** with a yield of 41%. m.p. 219.7–220.9 °C. ¹H NMR (300 MHz, CDCl₃) δ 8.87 (s, 1H), 8.20 (s, 1H), 7.88 (d, 1H, *J* = 8.1 Hz), 7.78 (s, 1H), 7.59–7.63 (m, 2H), 7.03 (s, 2H), 3.90–3.95 (m, 12H), 2.32 (s, 3H). HRMS: [M+H]⁺ *m/z* calcd for C₂₃H₂₄NO₆⁺: 410.1604, found: 410.1607. HPLC analysis: retention time = 9.4 min; peak area, >95% (254 nm).

5.1.24. (E)-3-(2-methyl-3-oxo-3-(3,4,5-trimethoxyphenyl)prop-1-en-1-yl)-1H-indole-6-carboxylic acid (24)

To a solution of methyl (E)-3-(2-methyl-3-oxo-3-(3,4,5-trimethoxyphenyl)prop-1-en-1-yl)-1H-indole-6-carboxylate (0.25 mmol, 102.3 mg) in methanol/water (5:1), added sodium hydroxide (0.75 mmol, 30 mg) and stirred at room temperature for 9 h. The resulting mixture was concentrated to remove Methanol. The residual aqueous solution was partitioned with ethyl acetate (10 mL). The combined aqueous extracts were acidified to pH 2 with 1 M HCl. The combined organic layers were washed with water (2 × 10 mL) and brine (20 mL) and dried over anhydrous MgSO₄ and evaporation of the solvent afforded compound **24** with a yield of 93%. m.p. 230.5–232.2 °C. ¹H NMR (300 MHz, CDCl₃) δ 8.87 (s, 1H), 8.20 (s, 1H), 7.88 (d, 1H, *J* = 8.1 Hz), 7.78 (s, 1H), 7.59–7.63 (m, 2H), 7.03 (s, 2H), 3.90–3.95 (m, 12H), 2.32 (s, 3H). ¹³C NMR (150 MHz, DMSO-*d*₆) δ 192.82, 168.57, 166.40, 162.68, 142.49, 135.75, 130.20, 129.03, 128.52, 127.71, 126.28, 106.52, 93.96, 91.45, 56.02. HRMS: [M+H]⁺ *m/z* calcd for C₂₂H₂₂NO₆⁺: 396.1447, found: 396.1452. HPLC analysis: retention time = 7.7 min; peak area, >95% (254 nm).

5.1.25. (E)-2-methyl-3-(1H-pyrrolo [2,3-*b*]pyridin-3-yl)-1-(3,4,5-trimethoxyphenyl)prop-2-en-1-one (25)

Compound **25** was prepared following a similar procedure to compound **1** with a yield of 41%. m.p. 221.3–222.9 °C. ¹H NMR (300 MHz, CD₃OD-*d*₄) δ 8.30 (s, 1H), 7.92–7.99 (m, 2H), 7.62 (s, 1H), 7.18–7.20 (m, 2H), 7.05 (s, 2H), 3.88 (s, 9H), 2.31 (s, 3H). HRMS: [M+H]⁺ *m/z* calcd for C₂₀H₂₁N₂O₄⁺: 353.1501, found: 353.1501. HPLC analysis: retention time = 4.8 min; peak area, >95% (254 nm).

5.1.26. (E)-2-methyl-3-(1H-pyrrolo [2,3-*c*]pyridin-3-yl)-1-(3,4,5-trimethoxyphenyl)prop-2-en-1-one (26)

Compound **26** was prepared following a similar procedure to compound **1** with a yield of 40%. m.p. 185.6–186.4 °C. ¹H NMR (300 MHz, CDCl₃) δ 10.35 (s, 1H), 10.05 (s, 1H), 8.40 (s, 1H), 7.98–8.00 (m, 1H), 7.78 (s, 1H), 7.52 (s, 1H), 7.23 (s, 1H), 7.02 (s, 1H), 3.90–3.95 (m, 9H), 2.00 (s, 3H). HRMS: [M+H]⁺ *m/z* calcd for C₂₀H₂₁N₂O₄⁺: 353.1501, found: 353.1501. HPLC analysis: retention time = 5.1 min; peak area, >95% (254 nm).

5.1.27. (E)-2-methyl-3-(1H-pyrrolo [3,2-*c*]pyridin-3-yl)-1-(3,4,5-trimethoxyphenyl)prop-2-en-1-one (27)

Compound **27** was prepared following a similar procedure to compound **1** with a yield of 36%. m.p. 225.0–226.0 °C. ¹H NMR (300 MHz, CDCl₃) δ 8.69 (s, 1H), 8.15 (d, 1H, *J* = 5.1 Hz), 7.59 (s, 1H), 7.42 (s, 1H), 7.34 (d, 1H, *J* = 5.7 Hz), 6.87 (s, 2H), 3.74–3.79 (m, 9H), 3.20 (s, 1H), 2.16 (s, 3H). HRMS: [M+H]⁺ *m/z* calcd for C₂₀H₂₁N₂O₄⁺: 353.1501, found: 353.1499. HPLC analysis: retention time = 8.2 min; peak area, >95% (254 nm).

5.1.28. (E)-2-methyl-3-(1H-pyrrolo [3,2-*b*]pyridin-3-yl)-1-(3,4,5-trimethoxyphenyl)prop-2-en-1-one (28)

Compound **28** was prepared following a similar procedure to compound **1** with a yield of 39%. m.p. 248.4–248.7 °C. ¹H NMR (300 MHz, CD₃OD-*d*₄) δ 8.34 (s, 1H), 7.87–7.96 (m, 2H), 7.42 (s, 3H), 7.24 (s, 1H), 7.00 (s, 1H), 4.38 (s, 1H), 3.86–3.89 (m, 6H), 2.31 (s, 3H). HRMS: [M+H]⁺ *m/z* calcd for C₂₀H₂₁N₂O₄⁺: 353.1501, found: 353.1505. HPLC analysis: retention time = 8.2 min; peak area, >95% (254 nm).

5.1.29. (E)-3-(imidazo[1,2-*a*]pyridin-3-yl)-2-methyl-1-(3,4,5-trimethoxyphenyl)prop-2-en-1-one (29)

To a solution of 1-(3,4,5-trimethoxyphenyl)propan-1-one (200 mg, 0.89 mmol) in sodium hydroxide solution (1.34 mL, 50 wt%) was added pyrazolo[1,5-*a*]pyridine-3-carbaldehyde (44.05 mg, 0.30 mmol) and piperidine (0.2 mL). After stirred at 95 °C for 48 h, the reaction mixture was concentrated and recrystallized in ethanol at –20 °C for 24 h to obtain compound **29** with a yield of 41%. m.p. 143.4–143.9 °C. ¹H NMR (300 MHz, CDCl₃) δ 8.09 (s, 1H), 7.99 (d, 1H, *J* = 6.6 Hz), 7.74 (d, 1H, *J* = 9.0 Hz), 7.32–7.38 (m, 2H), 7.26 (s, 1H), 6.93–6.99 (m, 2H), 3.89–3.95 (m, 9H), 2.39 (s, 3H). ¹³C NMR (150 MHz, DMSO-*d*₆) δ 197.27, 196.75, 153.10, 146.87, 137.24, 134.07, 133.90, 126.51, 125.12, 123.35, 121.75, 118.57, 113.98, 106.96, 61.16, 56.50, 16.14. HRMS: [M+H]⁺ *m/z* calcd for C₂₀H₂₁N₂O₄⁺: 353.1501, found: 353.1506. HPLC analysis: retention time = 22.1 min; peak area, >95% (254 nm).

5.1.30. (E)-2-methyl-3-(pyrazolo [1,5-*a*]pyridin-3-yl)-1-(3,4,5-trimethoxyphenyl)prop-2-en-1-one (30)

Compound **30** was prepared following a similar procedure to compound **2** with a yield of 53%. m.p. 158.5–160.4 °C. ¹H NMR (300 MHz, CDCl₃) δ 8.48 (s, 1H), 7.56 (d, 1H, *J* = 3 Hz), 7.30 (s, 1H), 7.15–7.17 (m, 1H), 7.03 (s, 2H), 6.82–6.84 (m, 1H), 6.76 (s, 1H), 3.91 (d, 9H, *J* = 12 Hz), 2.39 (s, 3H). HRMS: [M+H]⁺ *m/z* calcd for C₂₀H₂₁N₂O₄⁺: 353.1501, found: 353.1499. HPLC analysis: retention time = 7.3 min; peak area, >95% (254 nm).

5.1.31. (E)-3-(benzo[b]thiophen-3-yl)-2-methyl-1-(3,4,5-trimethoxyphenyl)prop-2-en-1-one (31)

To a solution of 1-(3,4,5-trimethoxyphenyl)propan-1-one (448 mg, 2 mmol) in sodium hydroxide solution (3.0 mL, 50 wt%) was added benzo [b]thiophene-3-carbaldehyde (162.2 mg, 1 mmol) and piperidine (0.4 mL). After stirred at 95 °C for 48 h, the reaction mixture was concentrated and recrystallized in ethanol at −20 °C for 24 h to obtain compound **31** with a yield of 54%. m.p. 120.4–121.0 °C. ¹H NMR (300 MHz, CDCl₃) δ 7.91 (s, 1H), 7.70 (d, 2H, *J* = 13.2 Hz), 7.44 (d, 3H, *J* = 14.1 Hz), 7.09 (s, 2H), 3.91–3.96 (m, 9H), 2.35 (s, 3H). ¹³C NMR (150 MHz, DMSO-*d*₆) δ 198.31, 153.04, 141.59, 139.52, 139.34, 138.84, 137.51, 133.57, 132.41, 131.25, 127.56, 125.19, 124.81, 123.03, 121.61, 107.24, 61.15, 56.44, 15.80. HRMS: [M+H]⁺ *m/z* calcd for C₂₁H₂₁O₄S⁺: 369.1161, found: 369.1160. HPLC analysis: retention time = 5.8 min; peak area, >95% (254 nm).

5.2. Biological methods

5.2.1. Cell cultures

HCT116 (human CRC cell line) and CT26 (mouse CRC cell line) cells were purchased from the cell bank of the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (SIBS, CAS) and cultured in McCoy's 5A (Gibco, 16600-082) and 1640 (Gibco, 11875-093) media, respectively. The culture medium was supplemented with 1% penicillin-streptomycin (HyClone, SV30010) and 10% fetal bovine serum (Gibco, 10091148). Cells were cultured in a 37 °C incubator (Thermo Fisher, USA) containing 5% CO₂.

5.2.2. Cell viability assay

HCT116 and CT26 cells (5 × 10³ cells per well) were seeded in 96-well plates and treated with different concentrations of FC116 for 24 h. The CCK-8 solution (Domino, Japan) was diluted to a concentration of 10% with serum-free medium, and the cells were incubated for 1.5 h. The IC₅₀ was calculated based on the absorbance at 450 nm. In addition, Calcein/PI cell viability and cytotoxicity assay kit were used to detect cell viability by incubating cells with Calcein AM at 37 °C for 25 min and PI for 5 min at room temperature. The cells were assayed at 490 nm excitation wavelength with a High Content Analysis System (PerkinElmer, USA) for 24 h after treatment with different concentrations of FC116.

5.2.3. Colony formation assay

HCT116 cells were seeded in 12-well plates overnight at the density of 1.5 × 10⁵ cells per well, and treated with different concentrations of FC116 for 24 h. After reseeding the cells at a density of 1000 cells per well in 6-well plates, the cells were cultured for two weeks, and the culture medium was changed every three days, followed by staining with crystal violet. The number of colonies was counted to assess the colony forming ability.

5.2.4. ROS measurement

The ROS assay kit (Beyotime, S0033S) was used to detect the change of ROS levels after treatment with FC116 in HCT116 and CT26 cells for 24 h. DCFH-DA was diluted by serum-free culture medium to a final concentration of 10 μM and incubated with the cells for 20 min in a 37 °C cell incubator, then the cells were analyzed with the High Content system and flow cytometry (Beckman, USA), respectively.

5.2.5. Mitochondrial membrane potential (MMP) measurement

Detection of MMP in CRC was performed using the Mitochondrial Membrane Potential Assay Kit (Beyotime, C2006). HCT116 and CT26 cells were incubated overnight in 96-well plates at a density of 1 × 10⁴ cells per well, and then the cells were treated with FC116. Then the CCCP (10 mM) provided in the kit was diluted to 10 μM with serum-free medium and incubated for 20 min in a 37 °C cell incubator, and the cells were analyzed for fluorescence intensity observed with a high-content

analysis system.

5.2.6. Cell metabolism assay

Oxygen consumption rate (OCR) was assessed using the Mito Stress Test Kit (Agilent, 103015-100) by seeding HCT116 cells (8 × 10³ cells per well) in Seahorse XF 96-well culture plates and incubating overnight until fully apposed. Then the cells were treated with different concentrations of FC116 for 24 h, followed by incubation with bicarbonate-free low-buffered assay medium containing 10 mM glucose, 1 mM pyruvate, 2 mM glutamine, and 5 mM HEPES for 1 h. Baseline calibration of the instrument was performed, and finally the program was set to add oligomycin, FCCP, and rosette cyanobacteria/antimycin A in order, by Seahorse Bioscience XF96 Extracellular Flow Analyzer (Agilent Technologies, USA) to detect OCR values.

5.2.7. Drug affinity responsive target stability (DARTS) assay

DARTS assays were used to detect the effect of FC116 on the enzymatic stability of tubulin. Lysates of HCT116 and CT26 cells were divided into two aliquots. The lysates were incubated with FC116 (5 mM) or DMSO for 2 h at room temperature. Then the lysates were aliquoted and incubated with pronase (Roche, 10165921001) at a ratio of 1:300, 1:500, 1:1000, 1:2000, 1:3000 for 30 min at room temperature, SDS-PAGE buffer was added and boiled for 10 min at 100 °C. Finally, the lysates were analyzed by Western blot.

5.2.8. Microscale thermophoresis (MST)

The binding affinity of FC116 to tubulin was assayed with Monolith NT. 115 (Nano Temper, GER). The cell lysates overexpressing tubulin were collected at 48 h after transfection of HEK 293T cells with wild-type or mutant EGFP-tubulin plasmids. The total fluorescence intensity of tubulin/EGFP was assayed before the formal experiment, and the cell lysate was diluted to the optimal intensity of drug binding to fluorescent protein in the binding reaction with standard buffers [50 mM HEPES (pH 7.5), 50 mM NaCl, 10 mM CaCl₂, 5 mM DTT and 0.05% Tween-20]. Subsequently, different concentrations of FC116 were diluted 16 times with the cell lysate in a 1:1 gradient. After incubation for 15 min at room temperature and protection from light, the lysates were loaded with MST NT. 115 standard glass capillaries and the program was set up for measurement.

5.2.9. Cellular thermal shift assay (CETSA)

The effect of FC116 on the thermal stability of tubulin was examined with CETSA. The cell lysate was incubated with FC116 (5 mM) and DMSO at room temperature for 1 h, respectively. Aliquot the mixture and heat for 5 min at the indicated temperatures 37 °C, 40 °C, 43 °C, 46 °C, 49 °C, 52 °C, 55 °C, 58 °C, 61 °C, 64 °C, 67 °C, 70 °C. After cooling at room temperature and centrifuging at high speed, the supernatant was collected, added to SDS-PAGE buffer, and boiled at 100 °C for 10 min, and then analyzed by Western blotting.

5.2.10. Western blot analysis

CRC cells treated with different concentrations of FC116 were collected and added to NP-40 buffer containing phosphatase inhibitor and protease inhibitor, lysed on ice for 30 min, and the total supernatant protein was quantified by high-speed centrifugation using BCA assay kit (Beyotime, P0010S), boiled at 100 °C for 10 min with SDS-PAGE buffer. The protein samples (20 μg/well) were separated and transferred to PVDF membrane (GE, 10600021). Then the PVDF membrane was incubated with the primary antibody overnight at 4 °C, washed three times with TBST for 10 min each time, and then incubated with the secondary antibody for 1 h at room temperature and washed three times with TBST. The protein expression was imaged by a gel imaging system (Tenon, China) and analyzed by Image J software (NIH, USA).

5.2.11. Immunofluorescence analysis

HCT116 and CT26 cells were seeded into confocal dishes at the

density of 5×10^4 cells per dish overnight and treated with 10 nM/20 nM FC116 at 5% CO₂ for 24 h. After discarding the medium and washing with PBS, the cells were then fixed with 4% paraformaldehyde for 30 min at room temperature. After incubating with the primary antibody (1:500) for tubulin and the fluorescently labeled secondary antibody, the cells were finally stained with Hoechst 33342 for 5 min. Then, the fluorescent images were captured with GE Delta Vision OMX SR (GE, USA).

5.2.12. Patient-derived organoids (PDOs) sensitivity test

The PDOs were inoculated in 48-well plates at the density of 100 organoids per well. After 3 days, the organoids grew to about 50–80 µm, then the different concentrations of FC116 were added to the organoids for another 3 days. At the end of this experiment, the live/dead cells were fluorescently stained, microscopically photographed and the area of live organoids (green fluorescence) was counted, and the concentration inhibition curves were plotted. The PDOs drug sensitivity test was commissioned from D1 Medical Technology.

5.2.13. Animal experiments on xenograft tumors

Male BALB/c nude mice purchased from Shanghai Slaughter Laboratory Animal Co., Ltd. at 5 weeks were housed under specific pathogen-free conditions. The animal experiments conformed to national and international guidelines and were approved by the Animal Experimentation Ethics Committee of Shanghai University of Traditional Chinese Medicine. A xenograft tumor mouse model was constructed by subcutaneously injecting (5×10^6 HCT116 cells in 100 µL PBS) into the right flank of mice. When the tumor volume reached 50–80 mm³, the mice were randomly divided into 4 groups (n = 8): control, PTX (7 mg/kg) and FC116 (1.5 and 3 mg/kg). PTX was injected intraperitoneally three times a week and FC116 was injected intraperitoneally once a day for 21 consecutive days. The body weight and tumor volume of mice were monitored every two days. The formula for tumor volume was calculated: volume (mm³) = length × (width²)/2.

5.2.14. Animal experiments on Apc^{Min/+} mice

Shanghai Model Organisms Center, Inc. provided Apc^{Min/+} mice. And the data provided by the company reached a maximum number of intestinal polyps when the mice reached 10 weeks of age. Experimental mice were randomly divided into 4 groups (n = 6): control, PTX (7 mg/kg), and FC116 (1.5 and 3 mg/kg). PBS or FC116 was injected intraperitoneally once a day and PTX was injected intraperitoneally three times a week. After 45 days of treatment, the number of intestinal polyps was counted and the intestines were collected for H&E testing.

5.2.15. Statistical analysis

Statistical analyses were performed by GraphPad Prism 8.0 software (La Jolla, CA, USA). Each experiment was performed and analyzed at least three times. Data were expressed as mean ± standard deviation (SD) from at least three independent experiments. Comparisons were performed using two-tailed Student's t-test. The values of $P \leq 0.05$ were considered statistically significant.

Author contributions

Weidong Zhang, Xin Luan, Chunlin Zhuang and Hongzhuang Chen are responsible for project conceptualization, article writing, review, funding acquisition and supervision. Xiaoxia Liu, Ye Wu Bolin Du, Lijun Zhang and Dong Lu were responsible for the experiments, data analysis and writing the paper. Jinmei Jin, Yichen Liu, Xinyi Chen and Jiayi Lin made figures and revised the paper.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

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